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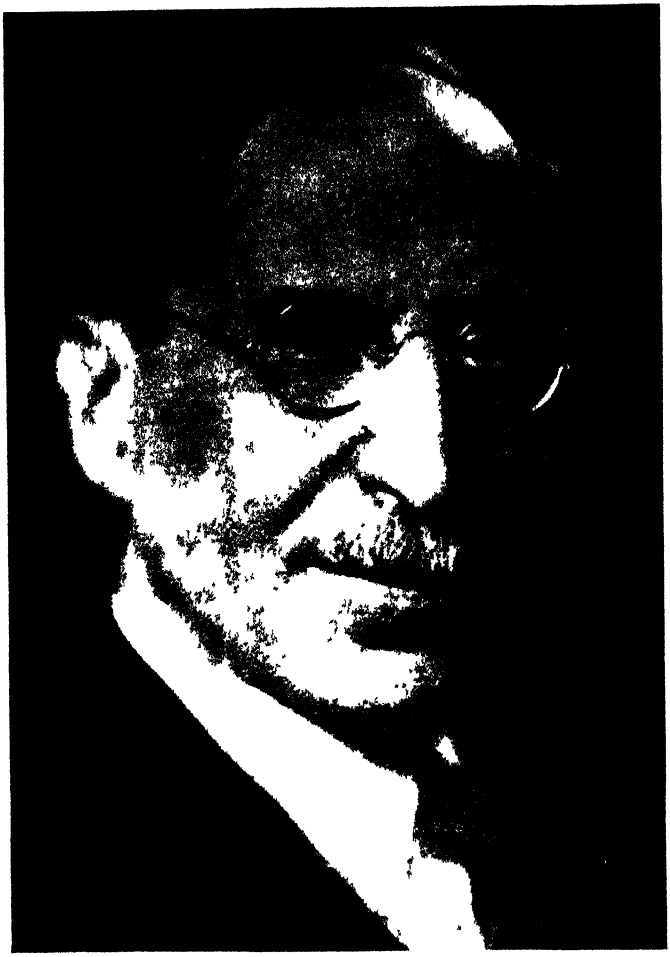

Milton Joseph Rosenau
1869–1946

Milton Joseph Rosenau

1869–1946

> "His life was gentle, and the elements
> So mix'd in him that Nature might stand up
> And say to all the world, 'This was a man!' "

Dr. Milton J. Rosenau, born in Philadelphia, Pennsylvania, on January 1, 1869, was graduated in medicine from the University of Pennsylvania at the age of twenty. He was commissioned in the U. S. Public Health Service, in which he remained from 1890 to 1909, and was Director of its Hygienic Laboratory from 1900 to 1909. From 1909 to 1935 he was Charles Wilder Professor of Preventive Medicine and Hygiene at the Harvard Medical School. In addition, he also served from 1914 to 1921 as Chief of the Division of Biologic Laboratories of the Massachusetts State Board of Health and from 1922 to 1935 as Professor of Epidemiology at the Harvard School of Public Health. From 1936 until his death he was Professor of Epidemiology and Dean of the School of Public Health at the University of North Carolina. He belonged to many scientific societies, and was awarded the Gold Medal of American Medicine in 1912-1913, the Sedgwick Medal in 1933, and a gold medal for outstanding contributions in allergy in 1945.

Few men have had such a full life as Dr. Rosenau. When the end came at the age of seventy-seven, he had been actively engaged in his chosen profession for fifty-seven years. Uppermost in his mind from the first was the prevention of the diseases of mankind. Even in his last publication he said, "If we want to preserve our way of life, we must preserve our health." He practiced this doctrine in his own extracurricular activities. He enjoyed practically all forms of sport both as spectator and as participant. It was his custom to take long walks in and around Boston. In tennis he was exceptionally good; in golf he was no "duffer"; and even in deck tennis he "threw a nasty ring." He liked the finest in music and drama. In social gatherings he was always gracious, dignified, and entertaining.

As an investigator he had the happy faculty of a straightforward approach. For example, his first publication (1900) on the characteristics of *Pasteurella pestis* indicated his realization of the need for fundamental information about the optimum conditions of growth and purity of the microorganism before study could be profitably extended to the prevention of the disease produced by the organism. In like manner, he saw the need for the development of methods in the use of disinfectants, antiseptics, and germicides, and therefore published his book, *Disinfection and Disinfectants; a Practical Guide for Sanitarians* (1902). In the same year he published, again in recognition of the need, a laboratory manual of bacteriology.

His many other studies while at the Hygienic Laboratory included the growth of the tubercle bacillus and other similar organisms using fruits and vegetables as a medium, and also research resulting in the establishment of the official unit for the standardization of diphtheria antitoxin and tetanus antitoxin. The study of anaphylaxis, in collaboration with Dr. J. F. Anderson, was a source of great satisfaction. He often said, in this connection, he was not sure that satisfaction came so much from the results obtained as from the hard work required. No subsequent work on this subject has been so comprehensive, including as it did the incubation period for sensitization, quantitative requirements for sensitivity and for shock, specificity, stability of sensitizer subjected to various chemical and physical conditions, and also the discovery that bacterial proteins were sensitizers. Further, he established the proper concentration of glycerol for the preservation of smallpox vaccine, with the consequent decrease of bacterial contamination, thus preventing infection following vaccination. Also he was in the forefront of those who advocated cleanliness and pasteurization to ensure a safe supply of milk, as is recorded in his book, *The Milk Question* (1912).

His acceptance of the Wilder Professorship at Harvard presented him with a new problem, a personal one—namely, how best to teach the subject of preventive medicine to medical students. It is obvious that, having spent the twenty years since graduation in the Public Health Service, he had had limited teaching experience. At the beginning this was compensated for by an intimate knowledge of public health measures gained from actually doing things himself. The practical application of a principle was never overlooked, and his students listened with rapt attention while he related anecdotes from his life in the Public Health Service to illustrate his points. Always the investigator applying the experimental method, he found that a brief answer in writing to a well-chosen question once a week measured the student's grasp of the subject and served as a guide for the clarification of his lectures. He also inaugurated the use of the sanitary survey as a practical means of teaching public health methods. Each student was required to select a community, make a detailed survey, and offer suggestions for improvements to a local official as well as in his written report. The outcome of his pedagogical research is indicated by polls conducted by the students to the effect that Dr. Rosenau was one of the best teachers at Harvard Medical School.

During his twenty-five years at Harvard he became more interested in the broad aspects of public health and preventive medicine. One of his great interests (1913–1922) was the establishment, in association with Drs. W. T. Sedgwick and G. C. Whipple, of the Harvard-Massachusetts Institute of Technology School for Health Officers. Later, at the opening of the Harvard School of Public Health, he was appointed Professor of Epidemiology. His research, however, continued on various subjects: a better milk supply, vitamins in milk, influenza and pneumonia, poliomyelitis, food poisoning, smallpox vaccine, and others. It is impossible in this short review to touch upon all the subjects investigated by Dr. Rosenau. With well over one hundred publications, including three books, his masterpiece is the textbook, *Preventive Medicine and Hygiene.* It has appeared in six editions, has been translated into Chinese, and is standard throughout the world as a textbook and encyclopedia on preventive medicine.

In his field Dr. Rosenau has not been excelled as an administrator. Always broad-minded, he appreciated the value of the individual. He aided independent research by those in his unit with his ready interest, his pertinent suggestions, his offers of any possible assistance to further the investigation, and, if deserved, a word of praise. One method of keeping his staff abreast of the times was the "Journal Club," which met at his home every month. It was here we learned the true greatness of the man.

As investigator, author, teacher, administrator, his influence was world-wide through his publications, his students, his associates, and his many addresses throughout this country and the rest of the world. "It is probable that no single individual has ever taught so many public health workers so much as M. J. Rosenau" (C.-E. A. Winslow).

His presidential address to the Society of American Bacteriologists, "Serendipity," gives in great part his philosophy on investigation. Like that of all great investigators, his formula was thought, work, and observation. Chance observations sometimes were important - serendipity—yet, as he stated, "The by-products of industry are often the most useful products, but we cannot have by-products unless we make products." With his thought always on the future, he said: "It is of no use to live longer unless we can live better. In other words, progress is measured in spiritual rather than material terms. Our next step, therefore, is mental and moral hygiene. Meanwhile, each of us adds his small piece to the mosaic of life, with the satisfaction that,

'When Earth's last picture is painted and the tubes are twisted
and dried,
. . . only the Master shall praise us, and only the Master shall blame;
And no one shall work for money, and no one shall work for fame,
But each for the joy of working, and each, in his separate star,
Shall draw the Thing as he sees it for the God of Things as They
are!' "

LLOYD D. FELTON

STUDIES ON THE NUTRITIONAL REQUIREMENTS OF BRUCELLA SUIS[1]

W. G. McCULLOUGH, R. C. MILLS, E. J. HERBST, W. G. ROESSLER,[2] AND
C. R. BREWER[3]

Received for publication September 7, 1946

Simplified media were first used for growth of *Brucella* by ZoBell and Meyer (1930, 1932), but such media produced only slight growth because the accessory growth factor requirements of this group were undetermined. These investigators, however, demonstrated utilization of the ammonium ion as a source of nitrogen and of various organic acids as sources of carbon and energy, and found that cystine was essential for growth, that iron salts were stimulatory, and that glycerol enhanced growth of *Brucella*. McNutt and Purwin (1931) obtained slight growth of some strains with xylose or arabinose as the sole carbon source. Glucose utilization was reported by Meyer and ZoBell (1932). Kerby (1939) showed that growth of *Brucella abortus* on Difco "tryptose" agar was enhanced by the addition of nicotinic acid and thiamine.

Koser, Breslove, and Dorfman (1941) first obtained abundant growth of *Brucella* in a chemically defined medium consisting of 17 amino acids, glucose, inorganic salts, thiamine, nicotinamide, biotin, and pantothenic acid. All the strains studied, including *Brucella melitensis*, *B. abortus*, and *B. suis*, required thiamine and nicotinamide (or nicotinic acid), and their growth was stimulated by pantothenic acid. Growth of the *B. abortus* strains was stimulated by a biotin concentrate. Koser and Wright (1942) showed later that the pyrimidine, but not the thiazole, component of thiamine is required by *Brucella*. N. B. McCullough and Dick (1942*a*, 1942*b*), using the basal medium of Koser *et al.* (1941), found that niacin was not required by *B. abortus*, that calcium pantothenate stimulated *B. suis* but had no effect on *B. abortus* and *B. melitensis*, and that biotin had no effect on *B. suis* or *B. melitensis*. They found that of 41 recently isolated strains of *B. abortus*, none grew on a synthetic medium, even with increased CO_2 tension. After acclimatization, 30 strains grew in a synthetic medium. All 30 strains required thiamine and biotin, 10 were stimulated by nicotinic acid, 11 by biotin, and 5 by both. These workers (1943) were able to obtain growth in a simple chemically defined medium with $(NH_4)_2SO_4$ as the sole source of nitrogen.

This paper presents the results of intensive studies of the nutritional requirements of one strain of *B. suis* (no. 1772-A obtained from Dr. I. F. Huddleson). A medium which gives growth 25 to 50 times as heavy as any previously reported on chemically defined media was developed. Eight other strains of *B. suis*, two of *B. abortus,* and one of *B. melitensis* were grown with comparable turbidities in the chemically defined media developed.

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[1] Work conducted at Camp Detrick, Frederick, Maryland, from February, 1945, to April, 1946.

[2] Lt., AUS, Ens., USNR; Ens., USNR, Lt. (jg), USNR; respectively.

[3] With the technical assistance of T/5 R. L. Liles, T/5 J. M. Fox, and F. B. Jones.

METHODS

Cultures were grown at 35 to 37 C in duplicate or triplicate in pyrex milk dilution bottles containing 10 ml of experimental medium. These bottles were shaken continuously on a reciprocating platform type shaker with a speed of 96 excursions per minute (4.5-inch stroke), providing uniform but not necessarily optimal aeration. Incubation periods ranged from 48 hours to several days. Growth was evaluated either turbidimetrically or by the poured plate technique. For turbidimetric measurements 1:10 dilutions were made in a diluent composed of 0.1 per cent tryptose and 0.5 per cent NaCl, and readings were made of the percentage of light transmission with a Coleman universal spectrophotometer, using a wave length of 650 mμ. When numbers of organisms were estimated by the poured plate technique, dilutions were made as accurately as possible in tryptose saline diluent, plated in triplicate on tryptose agar, and the plates incubated 72 hours at 37 C. The bacterial counts reported are averages of triplicate plates of two or more distinct cultures. All tests were repeated until confirmatory results were obtained.

Pyrex glassware, chemically cleaned and thoroughly rinsed with glass-distilled water, was used in preparing media and in growing cultures. Cotton plugs were enclosed in gauze to prevent fibers falling into cultures. All chemicals were the best grade commercially available. A solution of each substance was prepared in glass-distilled water, and appropriate amounts were used in preparing media. Medium constituents were combined in the culture bottles, made to volume, and sterilized by autoclaving 20 minutes at 15 pounds' pressure. The initial pH was 7.0.

Stock cultures were carried on tryptose agar slopes. Several slope cultures were prepared and incubated 48 hours. These cultures were refrigerated, and transplants from them were used to prepare inocula for experimental media. A return to the original stock cultures was made once a month. In the preparation of inocula, growth from a 24-hour tryptose agar slope culture was carefully removed and suspended in 0.5 per cent saline. The percentage of light transmission of the resultant cell suspension was determined and the suspension diluted so that one drop per culture bottle gave an inoculum of 3 to 5 $\times$ 10^5 organisms per ml. In earlier work (tables 1 and 2) inocula of about 1 $\times$ 10^6 cells per ml were used. This size of inoculum allowed maximum growth to occur in 48 to 52 hours, compared to 72 hours or more with the smaller inoculum. Cultures were checked microscopically for purity and the presence of a capsule.

RESULTS

The first experiments with chemically defined media were attempts to grow *B. suis* on the simplified medium of McCullough and Dick (1942*a*) and on the amino acid medium of Koser *et al.* (1941). No growth was noted in the simplified medium after 7 days, but a yield of 3.8 $\times$ 10^9 cells per ml was obtained in the amino acid medium after 7 days. Since much greater yields were obtained in simultaneous experiments with crude media, attempts were made to develop casein hydrolyzate media giving comparable yields and then to study the amino acid requirements of the organism.

The basic medium used in most of this work, devised partly from those described in previous publications, was as follows: 0.8 per cent NaCl, 0.0008 M (0.1 per cent) $MgSO_4$, 0.0057 M (0.1 per cent) K_2HPO_4 (Koser *et al.*, 1941), 4 ppm Fe^{++} (ZoBell and Meyer, 1932), 2 ppm Mn^{++}, 2 ppm Ca^{++}, 2 ppm Zn^{++}, 1 ppm Cu^{++}, 1 ppm Cd^{++}, 1 ppm Co^{++}, 1 ppm Ni^{++}, 0.1 per cent glycerol, and 1.0 per cent glucose. Niacin, thiamine, and calcium pantothenate, at 0.2 μg per ml each, were included in the early experiments until the actual requirements for them could be determined.

TABLE 1

Effect of various levels of glucose on growth of Brucells suis

MEDIUM	CELLS/ML
	billions
(1) 2% pepticase + 0.06% marmite + 0.5% NaCl + 0.5% glucose	19
(2) As (1) with 0.75% glucose	59
(3) As (1) with 1.00% glucose	84
(4) As (1) with 1.25% glucose	64
(5) As (1) with 1.50% glucose	47

TABLE 2

Effect of glycerol and inorganic ions on growth of Brucella suis

SUPPLEMENT ADDED TO MEDIUM*	CELLS/ML
	billions
(1) None	18
(2) 0.1% glycerol	16
(3) Inorganic salts†	41
(4) Inorganic salts + 0.1% glycerol	65
(5) Inorganic salts + 0.2% glycerol	68
(6) Inorganic salts + 0.4% glycerol	62
(7) 2 ppm Mn^{++} + 0.1% glycerol	69

* Medium: 1.0% glucose; 0.8% NaCl; 0.1% $MgSO_4$; 0.1% K_2HPO_4; 4 ppm Fe^{++}; 1.0% H_2SO_4-hydrolyzed "vitamin-free" casein; 0.2 μg/ml each of thiamine, calcium pantothenate, and niacin; 0.0002 M cystine; 0.002 M glycine; and 0.18% (dry weight) plasmolyzed yeast.

† Inorganic salts: 2 ppm Mn^{++}, Ca^{++}, Zn^{++}, 1 ppm Cu^{++}, Co^{++}, Cd^{++}, and Ni^{++}.

In preliminary work with crude media, using "pepticase"[4] as a nitrogen source, high levels of glucose increased the yields greatly. The optimal glucose concentration was found to be 1.0 per cent (table 1), and this level was used in all subsequent work. The addition of 0.1 to 0.2 per cent (dry weight) "plasmolyzed yeast"[5] or "marmite"[6] to the basal medium plus 1.0 per cent H_2SO_4-hydrolyzed "vitamin-free" casein resulted in excellent growth (tables 1 and 2).

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[4] A pancreatic digest of casein made by Sheffield Farms Co., Inc., New York, N. Y.

The effects of glycerol and inorganic salts on the growth of *Brucella suis* in a casein hydrolyzate medium are shown in table 2. Glycerol alone was ineffective, but in the presence of 2 ppm Mn^{++} large increases in yields were observed. The accessory relationship of Mn^{++} to glycerol stimulation was later found to be much less apparent in chemically defined media. Although Fe^{++} and Mn^{++} apparently replaced the inorganic salts mixture completely in this experiment, the complete mixture was used in subsequent work to assure adequacy of inorganic ions in more highly purified media.

In order to facilitate further work it was decided to use turbidity measurements of the cultures, rather than the laborious plate counts. It was first necessary, however, to show that the plate count could be correlated with the turbidity of the culture. Figure 1, with the percentage of light transmission of 1:10 dilutions of the cultures plotted against the viable cells per ml of original culture, shows satisfactory correlation between the two methods of evaluation. This was true only if the plate counts were made while the culture was still growing rapidly but was approaching its maximum turbidity. If the culture was shaken very long after maximum growth was attained, the number of viable cells decreased markedly. The data for the later experiments are all given as percentage of light transmission; plate counts were made at intervals as checks, and were satisfactory. The turbidities given in different tables cannot be compared

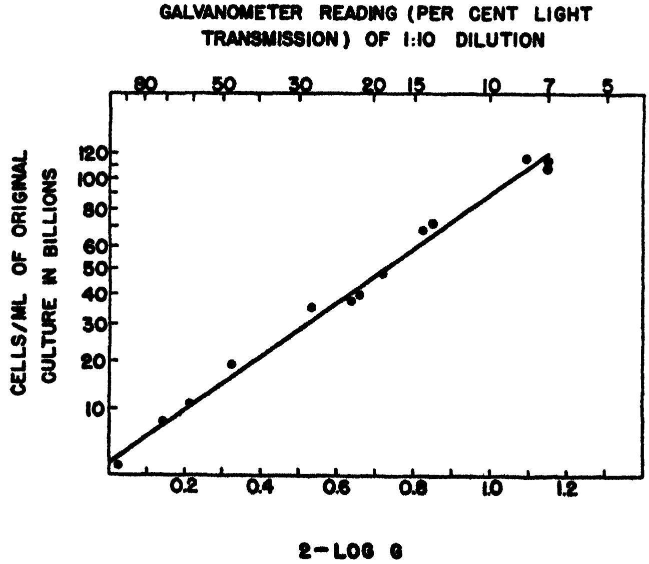

FIG. 1. CORRELATION OF CELL COUNTS AND TURBIDITIES OF BRUCELLA SUIS CULTURES

directly, as they were taken at various time intervals to show the differences under discussion. The data in each individual table were, of course, all taken at the same time, or as indicated, and are directly comparable. Figure 1 is presented to facilitate conversion of the percentage of light transmission given in tables 3 to 7 to cell counts.

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[5] An autolysed yeast product from ViCo Products Co., Inc., Chicago, Ill.

[6] A British autolysed yeast product.

The vitamin requirements on the casein hydrolyzate medium were ascertained, and adequate levels of the four vitamins found to be required (thiamine and niacin) or stimulatory (biotin and calcium pantothenate) were used in the determination of amino acid requirements. When a satisfactory amino acid mixture was available, the levels of each vitamin needed for maximum growth were redetermined. Figure 2 shows the curves obtained with thiamine and niacin.

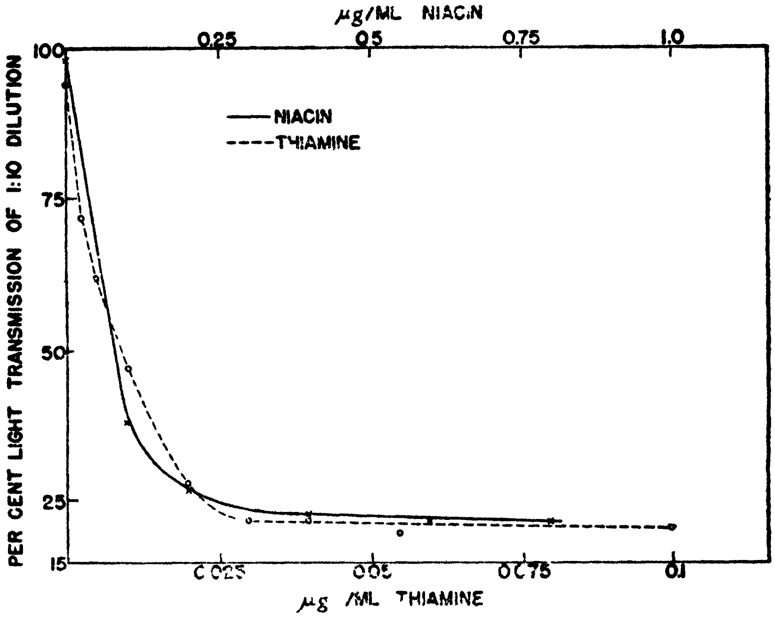

FIG. 2 EFFECTS OF VARYING CONCENTRATIONS OF NIACIN AND THIAMINE ON GROWTH OF BRUCELLA SUIS

It can be seen that about 0.03 µg per ml thiamine and 0.4 µg per ml niacin allowed maximal growth of the organism under the conditions of the experiment. Niacinamide had approximately the same activity as niacin. Table 3 shows the growth obtained at various levels of biotin and calcium pantothenate. The levels of both vitamins required for the most rapid growth were higher than the levels needed for maximum growth after a longer time. Levels of 0.0001 µg per ml biotin and 0.1 µg per ml calcium pantothenate allowed the most rapid growth, but 0.00001 µg per ml biotin and 0.01 µg per ml calcium pantothenate gave the same maximum growth after 96 hours as did the higher levels (see also table 7).

The amino acid mixture of Koser *et al.* (1941) was modified to give considerably better growth, and the resulting mixture was used for determination of the essentiality of individual amino acids. The results of the omission of single amino acids from the complete mixture, as well as their concentrations in the complete medium, are given in table 4. Cystine, histidine, tyrosine, phenylalanine, and tryptophane were essential, little or no growth occurring in their absence. Glycine, lysine, arginine, methionine, and glutamic acid were stimulatory but not essential. Alanine and serine were slightly toxic at the concentrations used, better growth occurring in their absence. A higher level of histidine markedly accelerated growth. It was found that a mixture of the 10 amino acids indicated by daggers in table 4 gave as good growth as the combination of 17 acids. Table 5 shows the effects of various additions to these 10 amino acids. Isoleucine and aspartic acid were both found to be stimulatory, and further increases in histidine were beneficial. A lower level of serine than that used in table 4 was stimulatory, rather than toxic, as was a lower level of threonine. The addition of plasmolyzed yeast or casein hydrolyzate caused rapid and heavy growth. The ability of casein hydrolyzate to produce as heavy growth as the plasmolyzed yeast indicates that the addition of the proper amino acids to the medium may be all that is necessary to produce maximal growth of the organism.

TABLE 3

Effect of biotin and calcium pantothenate on growth of Brucella suis

MEDIUM	LIGHT TRANSMISSION OF 1:10 DILUTION	
	70 hr	96 hr
	per cent	*per cent*
(1) Basal* minus biotin	88	64
(2) As (1) + 0.00001 μg/ml biotin	49	17
(3) As (1) + 0.0001 μg/ml biotin	38	16
(4) As (1) + 0.001 μg/ml biotin	38	16
(5) Basal minus calcium pantothenate	90	44
(6) As (5) + 0.001 μg/ml calcium pantothenate	80	25
(7) As (5) + 0.003 μg/ml calcium pantothenate	68	18
(8) As (5) + 0.01 μg/ml calcium pantothenate	46	16
(9) As (5) + 0.03 μg/ml calcium pantothenate	40	15
(10) As (5) + 0.10 μg/ml calcium pantothenate	34	15
(11) As (5) + 0.30 μg/ml calcium pantothenate	35	16

* Basic medium constituents plus 0.8 μg/ml niacin, 0.1 μg/ml thiamine, 0.3 μg/ml calcium pantothenate, 0.001 μg/ml biotin, and the 17 amino acids in table 4.

Although the addition of casein hydrolyzate to the amino acid medium caused maximal rapid growth, yeast nucleic acid or some of its components stimulated growth in the absence of the casein hydrolyzate. Little or no difference in final yield was noted, but the nucleic acid caused definite acceleration of growth (tables 6 and 7). Equivalent amounts of "N" mixture, a combination of uracil, adenine, guanine, cytosine, and ribose in the proportions found in nucleic acid and in the amounts given in table 6, had the same activity as the nucleic acid. Adenine, guanine, and cytosine also completely replaced, and uracil nearly replaced, the nucleic acid. It was later found that 0.000005 M "N" mixture was as active as 0.0001 M.

The effects of inorganic ions on the growth of *Brucella suis* were studied in a chemically defined medium (the amino acid medium shown in table 4). Removal of Mg^{++} prevented growth; Mg^{++} in concentrations from 0.0001 M to 0.003 M gave maximal growth. Removal of Fe^{++} reduced yields from 70×10^9 cells per ml to less than 20×10^9 cells per ml. Fe^{++} from 0.1 ppm to 10 ppm gave maximal growth; higher levels of Fe^{++} were inhibitive. (The iron content of this basal medium, with the *ortho*-phenanthroline method, was less than 0.05 ppm. Hydrolyzed casein basal media contained less than 2 ppm iron.) The optimal concentration of K_2HPO_4 was found to be 0.1 per cent. Added K^{+} was harmful. The presence of Mn^{++} and Ca^{++} did not affect the final yield, but

TABLE 4

Effect of omission of single amino acids from complete mixture on growth of Brucella suis

AMINO ACID OMITTED FROM MEDIUM[*]	FINAL MOLARITY IN COMPLETE MEDIUM	LIGHT TRANSMISSION OF 1:10 DILUTION
		per cent
None		54
Glycine[†]	0.0045	75
dl-Alanine	0.006	38
dl-Valine	0.001	48
dl-Leucine	0.0008	53
dl-Lysine[†]	0.001	78
l(+)Arginine[†]	0.000475	64
dl-Serine	0.0015	40
dl-Threonine	0.001	55
dl-Glutamic acid[†]	0.0028	57
l(−)Cystine[†]	0.0008	95
dl-Methionine[†]	0.0007	69
l(−)Histidine[†]	0.0036	96
l(−)Tyrosine[†]	0.00027	90
dl-Phenylalanine[†]	0.0006	94
l(−)Proline	0.0009	48
l(−)Hydroxyproline	0.0008	44
l(−)Tryptophane[†]	0.0001	96
None omitted, with histidine doubled	0.0072	28

[*] Basic medium constituents plus 0.8 μg/ml niacin, 0.1 μg/ml thiamine, 0.3 μg/ml calcium pantothenate, 0.001 μg/ml biotin, and the 17 amino acids listed above used in the concentrations indicated.

[†] These amino acids were considered essential or stimulatory, and were used in succeeding experiments in the concentrations indicated above.

0.1 ppm Mn^{++} and 1.0 ppm Ca^{++} stimulated early growth. Cu^{++} was toxic above 2.0 ppm, but 5 ppm of Fe^{++}, Ca^{++}, Mn^{++}, Cd^{++}, Co^{++}, Zn^{++}, or Ni^{++} had no adverse effect. The absence of Cd^{++}, Co^{++}, Zn^{++}, and Ni^{++} did not affect the rate of growth.

TABLE 5

Effect of addition of various amino acids to improved chemically defined medium

ADDITION TO MEDIUM*	LIGHT TRANSMISSION OF 1:10 DILUTION
	per cent
(1) None	52
(2) 0.00045 M *dl*-isoleucine	39
(3) 0.005 M *dl*-aspartic acid	44
(4) 0.00045 M isoleucine + 0.005 M aspartic acid	36
(5) As (4) + 0.0036 M histidine	36
(6) As (4) + 0.0072 M histidine	31
(7) As (4) + 0.0144 M histidine	23
(8) As (4) + 0.0288 M histidine	20
(9) As (6) + 0.00015 M serine	24
(10) As (6) + 0.0006 M serine	28
(11) As (6) + 0.0001 M threonine	28
(12) As (6) + 0.0004 M threonine	27
(13) As (6) + 0.6% casein hydrolyzate	8
(14) As (6) + 0.18% plasmolyzed yeast	8

* Basic medium constituents plus 0.8 μg/ml niacin, 0.1 μg/ml thiamine, 0.3 μg/ml calcium pantothenate, 0.001 μg/ml biotin, and the 10 essential and stimulatory amino acids indicated in table 4.

TABLE 6

Effect of yeast nucleic acid and its constituents on growth of Brucella suis

ADDITION TO MEDIUM*	LIGHT TRANSMISSION OF 1:10 DILUTION
	per cent
None	32
0.005% yeast nucleic acid	19
0.00005 M "N" mixture	18
0.00005 M uracil	24
0.00005 M adenine	19
0.00005 M guanine	20
0.00005 M cytosine	19
0.005% yeast nucleic acid + 0.6% casein hydrolyzate	8
0.6% casein hydrolyzate	8

* Basic medium constituents plus 0.8 μg/ml niacin, 0.1 μg/ml thiamine, 0.3 μg/ml calcium pantothenate, 0.001 μg/ml biotin, the 10 essential and stimulatory amino acids in table 4, 0.0144 M histidine, 0.005 M aspartic acid, and 0.00045 M isoleucine.

Table 7 shows the effects of decreasing the size of the inoculum on the stimulations by yeast nucleic acid, biotin, and calcium pantothenate. With an inoculum of 16,000 cells per ml the complete medium almost reached maximum growth at 4 days, so no stimulation by nucleic acid was evident. With 560 cells per ml growth was somewhat accelerated by the yeast nucleic acid, but with 16 cells per ml the nucleic acid caused marked stimulation of growth, maximum growth being attained 24 hours earlier. The inoculum of 16,000 cells per ml allowed maximum growth in 6 days in the media without biotin and calcium pantothenate, but with smaller inocula no growth was evident in 6 days without biotin, and growth without calcium pantothenate was just beginning in 6 days.

The culture was carried through 10 serial transfers in the amino acid medium used in table 4 with no decrease in yield or change in cultural characteristics.

B. suis cultures grown in chemically defined media were just as infective as those grown in natural media, when dilutions were injected into albino mice together with mucin solutions, using the method of Spencer and Kelly (1945).

TABLE 7

Effect of size of inoculum on stimulation of Brucella suis by biotin, calcium pantothenate, and yeast nucleic acid

INOCULUM	INCUBATION TIME	LIGHT TRANSMISSION OF 1:10 DILUTION			
		Complete medium*	Complete minus biotin	Complete minus calcium pantothenate	Complete plus 0.005% yeast nucleic acid
cells/ml	*days*	*per cent*	*per cent*	*per cent*	*per cent*
16,000	4	22	98	97	20
	5	18	84	54	17
	6		22	24	
560	4	67	98	98	49
	5	23	98	90	21
	6	20	96	41	
16	4	96	98	98	62
	5	50	98	96	21
	6	21	94	67	

* Basic medium constituents plus 17 amino acids (table 4), 0.8 μg/ml niacin, 0.1 μg/ml thiamine, 0.3 μg/ml calcium pantothenate, and 0.001 μg/ml biotin.

In a brief survey of the vitamin requirements of other strains, 13 *Brucella* strains (8 *B. suis*, 3 *B. abortus*, and 2 *B. melitensis*) were grown on the amino acid medium, complete and with various omissions. Inocula of about 20,000 cells per ml were used. All 13 strains had absolute requirements for thiamine and niacin. With this level of inoculum, none of the *B. suis* strains required biotin, but all of the *B. abortus* and *B. melitensis* strains required it. One strain of *B. melitensis* and one of *B. abortus* did not grow except when plasmolyzed yeast was added to the medium. Four strains of *B. suis*, two of *B. abortus*, and one of *B. melitensis* were stimulated by calcium pantothenate. It is quite possible that, with smaller inocula, more strains would have been stimulated by biotin and calcium pantothenate.

Several practical media were developed which routinely gave yields of more than 100 × 10^9 cells per ml. The addition of 1.0 per cent glucose, 0.1 μg per ml thiamine, and 4 ppm Fe^{++} to ordinary tryptose broth (2.0 per cent tryptose, 0.5 per cent NaCl), ordinarily used for growth of *Brucella*, increased yields (with good aeration) from about 10 × 10^9 cells per ml to 70 to 80 × 10^9 cells per ml. Even better results were obtained when tryptose was replaced by 2.0 per cent pepticase plus 0.12 per cent plasmolyzed yeast. The time of incubation required for maximum growth depended on the size of the inoculum.

DISCUSSION

The results presented here confirm the early observations of ZoBell and Meyer (1930, 1932) that cystine is essential, and that iron salts and glycerol are stimulatory, to *Brucella*. In agreement with the work of Koser *et al.* (1941), all strains studied required thiamine and niacin, and many were stimulated by calcium pantothenate. Neither Koser *et al.* (1941) nor McCullough and Dick (1942*a*) found stimulation of *B. suis* by biotin, although we noted a marked stimulation by it when small inocula were used, and when all other nutritional requirements were met. The requirement of this strain of *B. suis* for niacin or niacinamide is unusually high, at least 0.4 μg per ml being required for maximum growth.

The yields obtained here are much greater than those previously reported on chemically defined media. Koser *et al.* (1941, 1942) obtained yields of 5 to 10 × 10^8 cells per ml and light transmission of 60 per cent, or about $\frac{1}{50}$ of the growth obtained here. The higher glucose levels used here account for part of the difference in yields, as an increase in glucose from 0.1 per cent to 1.0 per cent causes 4- to 5-fold increases in yields, when other requirements are also met.

An interesting but unexplained difference was observed in the effect of manganese salts on growth in media containing crude substances such as plasmolyzed yeast in which manganese was markedly stimulatory, and chemically defined media in which much less effect was found.

Yeast nucleic acid or its component parts were markedly stimulatory to growth, especially with small inocula. Unfortunately, this phase of the work could not be pursued further, because of discontinuation of the project; this is also true of efforts to identify the substances in casein hydrolyzate that stimulate growth on the best chemically defined medium developed.

The best amino acid mixture developed differed from that of Koser *et al.* (1941) in that it contained isoleucine and aspartic acid, and did not contain alanine, hydroxyproline, proline, valine, or leucine. Increasing amounts of histidine stimulated growth until a level 24 times that used by Koser *et al.* (1941) was reached. The active levels of histidine are unusually high; its activity may not be specific.

Application of the knowledge of the nutritional requirements of the organism resulted in increasing the yields in tryptose broth 5 to 10 times merely by the addition of appropriate concentrations of glucose, thiamine, and iron, with adequate aeration.

SUMMARY

The nutritional requirements of a strain of *Brucella suis* were intensively studied, and a chemically defined medium was devised which gave yields greater than those previously obtained in any medium.

Thiamine (0.03 μg per ml) and niacin (0.4 μg per ml) were essential for growth, and biotin (0.0001 μg per ml) and calcium pantothenate (0.1 μg per ml) were stimulatory, especially when small inocula were used.

Yeast nucleic acid or any of its component pyrimidines and purines stimulated early growth.

Cystine, histidine, tyrosine, phenylalanine, and tryptophane were essential for growth, whereas glycine, lysine, arginine, methionine, glutamic acid, isoleucine, aspartic acid, serine, and threonine were stimulatory.

Magnesium salts were essential, and manganese and iron salts were stimulatory.

One per cent glucose was required for maximum growth.

The addition of glucose, thiamine, and iron salts to "tryptose" broth increased yields 5- to 10-fold.

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PENICILLIN ASSAY TECHNIQUES: A COMPARATIVE STUDY[1]

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Since the original cup method for penicillin assay was described by Fleming (1942), numerous modifications of procedure have been suggested by other groups of investigators as well as by Fleming (1944). (See Foster and Woodruff, 1943; Cooke, 1945; Beadle *et al.*, 1945; Woolley and Schmidt, 1945; Asheshov and Strelitz, 1945.) Joslyn (1944) has described a turbidimetric assay procedure that has been used by some groups. It is the general consensus that both the cylinder plate method and the turbidimetric method are of doubtful reliability and sensitivity for the assay of penicillin in blood. In the cylinder plate method the presence of a large amount of inactive solids in blood seems to interfere with the proper diffusion of penicillin. Difficulties arise in the turbidimetric method because the medium is not of the same composition throughout the series. This discrepancy would require the use of a constant amount of blood plasma in each tube while the procedure is being carried out in order that the nutritional variable may become constant.

Rammelkamp and Keefer (1942) described a more practical serial dilution method for penicillin assay based on the hemolysis of erythrocytes by a standard strain of group A hemolytic streptococcus, and this method has been in common use. Further modification of this procedure, again employing a standard strain of streptococcus, *Streptococcus pyogenes* C-203, was described by Rosenblatt *et al.* (1944).

For large-scale rapid assays, the serial dilution method has been modified by the Food and Drug Administration and has been adopted by this body for routine assay purposes. This method, described by Randall, Price, and Welch (1945), uses *Bacillus subtilis* NRRL B-558 as the test organism. Since this assay is based on the reading of the growth of the organism as against complete inhibition, the nutritional effect of the blood plasma is minimized. The chance presence of small amounts of erythrocytes or leucocytes produces no great effect since the test organism *Bacillus subtilis* is nonhemolytic in contrast to other test organisms. Usually red and white cell components settle to the bottom of the tube during the period of incubation and do not cause turbidity of the supernatant liquid unless the tubes have been agitated.

In the setting up of assay procedures in the Third Medical Service Laboratory at St. Luke's Hospital, the Rosenblatt *et al.* modification of the Rammelkamp and Keefer procedure was initially employed. It was observed that there was a considerable variation in results despite careful checking and rechecking of accepted technical procedures. It was finally suggested that the FDA assay procedure be adopted.[3] With this method the consistency of results has been most satisfactory, and no technical problems have arisen. We then attempted to determine the reasons for the great variance in results obtained by the two methods.

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[1] These studies were aided by a grant from the Commercial Solvents Corporation, New York, N. Y.

It should be indicated at the outset that blood assays have been conducted by us on well over 150 subjects. We are aware of the objection to *Bacillus subtilis* assay techniques voiced by Elias *et al.* (1945) and Chandler *et al.* (1945) on the grounds that patients frequently contain antibody for *Bacillus subtilis*, thus causing inhibition of growth of the test organism not due to penicillin activity. This observation was not borne out in our studies, and in no instance did the control tubes show inhibition.

With blood samples from patients receiving various doses and kinds of penicillin, 39 assays were performed using both the *Streptococcus pyogenes* C-203 and *Bacillus subtilis* NRRL B-558 assay procedures. The results (34 samples) averaged 53 per cent lower with *Streptococcus pyogenes* as the test organism. In the remaining 5 samples no levels were obtained with *Streptococcus pyogenes* as the test organism, but levels of 0.06 units per ml were obtained with the *Bacillus subtilis* procedure. These samples were assayed in our own laboratory, and, in addition, aliquot portions were checked by the *Streptococcus pyogenes* assay procedure in the laboratories of the Chicago Intensive Treatment Center of the Chicago Health Department co-operating with the United States Public Health Service. With these data available, further studies were undertaken.

In dealing with biologic assay methods it is necessary to re-emphasize that we are dealing with complicated growth systems. It was believed reasonable that these systems should be assayed as an environmental whole, and with this in mind we wondered concerning the appropriateness of using a penicillin-saline solution as a standard test solution, as described by Rosenblatt *et al.*, when assaying a penicillin-serum system in the unknown. Comparative studies were then run using standards made with penicillin in normal human serum instead of saline solution in an effort to explain the difference in values obtained when *Streptococcus pyogenes* is used as the test organism. The exact procedures used in conducting the two assay techniques are indicated in table 1.

In both techniques the standard solutions are made up in saline solution. A comparison of the end point—complete inhibition—in the dilutions of the standard solution and the dilutions of the unknown gives the units of penicillin in the sample being tested. Since this comparison is the basis of the calculated result, it is important that the sensitivity of the test organism to penicillin should not vary in a saline broth and a serum broth environment. It was found that the sensitivity of *Bacillus subtilis* to penicillin in a saline broth and in serum broth environment is statistically the same, whereas the sensitivity of *Streptococcus pyogenes* to the two media differs widely (table 2).

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[3] We are greatly indebted to Dr. Richard J. Hickey, biochemist of the Commercial Solvents Corporation, for his numerous suggestions and personal aid in standardizing our assay procedures.

TABLE 1

Comparison of techniques

ORGANISM	*Streptococcus pyogenes* (C-203)	*Bacillus subtilis* (NRRL B-558)
Medium	Brain heart broth	Schmidt-Moyer yeast beef broth
Culture used	2 ml 6-hour broth culture in 100 ml	10 ml 24-hour broth culture grown under oil layer in 100 ml
Material used	Serum	Plasma
Standard	0.1 unit per ml in saline	1 unit per ml in saline
Preparation of equipment	Individual tubes plugged	Racks and tubes may be wrapped and sterilized without plugging
Dilution method	0.1 ml serum difference in each tube	Serial
Pipetting	By hand	Brewer pipetting machine may be used
Incubation	37 C for 18 hours	37 C for 18 to 24 hours
End point	Tube without turbidity	Tube without turbidity
Sensitivity of organism (in serum)	0.026 units ±0.001	0.014 units ±0.002

TABLE 2

Sensitivity of Bacillis subtilis and Streptococcus pyogenes to penicillin in saline broth and serum broth

(Technique involved)

Working standard—0.1 units per ml (saline/serum)

Amount of standard saline serum (ml)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
Broth (ml)	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
Concentration of penicillin (u/ml)	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
Infected broth	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Final concentration of penicillin (u/ml)	0.003	0.0066	0.0100	0.0133	0.0166	0.0200	0.0233	0.0266	0.0300

	B. subtilis NRRL B-558		*S. pyogenes* C-203	
	Saline broth	Serum broth	Saline broth	Serum broth
	u/ml	*u/ml*	*u/ml*	*u/ml*
	0.0166	0.020	0.0066	0.020
	0.0166	0.013	0.0066	0.0266
	0.0166	0.013	0.010	0.0266
	0.020	0.013	0.0066	0.0266
	0.020	0.013	0.0066	0.0266
	0.020	0.013	0.0066	0.0266
	0.013	0.013	0.0066	0.0266
	0.013	0.013	0.010	0.0266
	0.013	0.013	0.010	0.0266
Mean	0.017	0.014	0.008	0.026
Mean deviation	±.002	±.002	±.002	±.001

TABLE 3

*Comparison of assay results using serum with a known (added) amount of penicillin**

KNOWN CONCENTRATION PENICILLIN	ASSAY USING *B. subtilis* NRRL B-558				ASSAY USING *S. pyogenes* C-203			
	Test runs	Assay	Mean $\frac{\Sigma X}{N}$	Mean deviation D/N	Test runs	Assay	Mean $\frac{\Sigma X}{N}$	Mean deviation D/N
units/ml								
0.01	1	0	0	0	1	0	0	0
	2	0			2	0		
	3	0			3	0		
	4	0			4	0		
	5	0						
	6	0						
	7	0						
	8	0						
0.03	1	0.03	0.015	±0.015	1	0	0	0
	2	0.03			2	0		
	3	0.03			3	0		
	4	0.0			4	0		
	5	0.03						
	6	0.0						
	7	0.0						
	8	0.0						
0.06	1	0.06	0.06	±0.	1	0	0	0
	2	0.06			2	0		
	3	0.06			3	0		
	4	0.06			4	0		
	5	0.06						
	6	0.06						
	7	0.06						
	8	0.06						
0.1	1	0.125	0.0925	±0.0325	1	0.05	0.04	±0.01
	2	0.06			2	0.05		
	3	0.06			3	0.05		
	4	0.06			4	0.05		
	5	0.125			5	0.03		
	6	0.125			6	0.03		
	7	0.125			7	0.03		
	8	0.06						
0.2	1	0.125	0.125	0	1	0.1	0.1	0
	2	0.125			2	0.1		
	3	0.125			3	0.1		
	4	0.125			4	0.1		
	5	0.125			5	0.1		
	6	0.125			6	0.1		
	7	0.125			7	0.1		
	8	0.125						

TABLE 3—*Continued*

KNOWN CONCENTRATION PENICILLIN	ASSAY USING *B. subtilis* NRRL B-558				ASSAY USING *S. pyogenes* C-203			
units/ml	Test runs	Assay	Mean $\frac{\Sigma X}{N}$	Mean deviation D/N	Test runs	Assay	Mean $\frac{\Sigma X}{N}$	Mean deviation D/N
0.3	1	0.25	0.2185	±0.0470	1	0.15	0.15	0
	2	0.25			2	0.15		
	3	0.25			3	0.15		
	4	0.25			4	0.15		
	5	0.125			5	0.15		
	6	0.125			6	0.15		
	7	0.25			7	0.15		
	8	0.25						
0.4	1	0.25	0.25	0	1	0.2	0.18	±0.023
	2	0.25			2	0.2		
	3	0.25			3	0.2		
	4	0.25			4	0.2		
	5	0.25			5	0.2		
	6	0.25			6	0.15		
	7	0.25			7	0.15		
	8	0.25			8	0.15		
0.5	1	0.5	0.343	±0.115	1	0.25	0.24	±0.01
	2	0.25			2	0.25		
	3	0.25			3	0.25		
	4	0.5			4	0.25		
	5	0.5			5	0.25		
	6	0.25			6	0.25		
	7	0.25			7	0.20		
	8	0.25						
0.6	1	0.5	0.5	0	1	0.35	0.32	±0.04
	2	0.5			2	0.35		
	3	0.5			3	0.35		
	4	0.5			4	0.25		
	5	0.5			5	0.25		
	6	0.5			6	0.35		
	7	0.5			7	0.35		
	8	0.5						
0.7	1	0.5	0.5	0	1	0.3	0.31	±0.018
	2	0.5			2	0.3		
	3	0.5			3	0.3		
	4	0.5			4	0.3		
	5	0.5			5	0.3		
	6	0.5			6	0.35		
	7	0.5			7	0.35		
	8	0.5						

TABLE 3—*Continued*

KNOWN CONCENTRATION PENICILLIN	ASSAY USING *B. subtilis* NRRL B-558				ASSAY USING *S. pyogenes* C-203			
	Test runs	Assay	Mean $\frac{\Sigma X}{N}$	Mean deviation D/N	Test runs	Assay	Mean $\frac{\Sigma X}{N}$	Mean deviation D/N
units/ml								
1.0	1	1.0	1.0	0	1	0.5	0.5	0
	2	1.0			2	0.5		
	3	1.0			3	0.5		
	4	1.0			4	0.5		
	5	1.0						
	6	1.0						
	7	1.0						
	8	1.0						
2.0	1	2.0	2 0	0	1	1.0	1.0	0
	2	2.0			2	1.0		
	3	2.0			3	1.0		
	4	2.0			4	1.0		
	5	2.0						
	6	2.0						
	7	2.0						
	8	2.0						
3.0	1	2.0	2.0	0	1	1.5	1.5	0
	2	2.0			2	1.5		
	3	2.0			3	1.5		
	4	2.0			4	1.5		
	5	2.0						
	6	2.0						
	7	2.0						
	8	2.0						
4.0	1	4 0	4.0	0	1	1.5	1.75	±0.25
	2	4.0			2	1.5		
	3	4.0			3	2.0		
	4	4.0			4	2.0		
	5	4.0						
	6	4.0						
	7	4.0						
	8	4.0						

* If the Rosenblatt *et al.* technique is standardized in a normal serum-penicillin environment, then the calculated result for unknown sera becomes almost an absolute value. For example: If, in table 3, the true end point value in serum-penicillin calculates to be in the 0.6 tube for 0.1 unit per ml concentration of penicillin in serum, then the assayed results should be determined against 0 06 units per ml, not 0.03 units per ml, as is found in a saline-penicillin environment. The tube tenths used should then become the denominator and 0.06 the numerator. In this particular set of runs, multiplying by the factor 2 would then yield a more accurate assay result.

Discussion of table 2. The differences involved in assays of identical sera tested by the Rosenblatt technique and that of the FDA assay procedure indicated the need for a comparison of the sensitivity of the two organisms used (*Bacillus subtilis* NRRL B-558 and *Streptococcus pyogenes* C-203) when exposed to a penicillin saline broth and a penicillin serum broth environment. This was accomplished by making two working standards: one containing *saline* with 0.1 of a unit of penicillin per ml and the other containing *serum* with 0.1 of a unit of penicillin per ml. Series of sensitivity tests with the two organisms were then run against the saline-penicillin and serum-penicillin standard mixtures.

Statistically, the growth of *Bacillus subtilis* NRRL B-558 did not change in the two environments as evidenced by the consistent inhibition in both cases in penicillin concentrations of 0.013 units per ml to 0.02 units per ml. However, the comparative values obtained in the two systems when using *Streptococcus pyogenes* C-203 indicates that the growth phase of this organism is drastically altered in a penicillin-serum environment. Penicillin concentrations in saline necessary for inhibition of this organism varied from 0.01 to 0.0066 units per ml, whereas the values obtained in the penicillin-serum environment necessitated as much as 0.02 units per ml to 0.0266 units per ml.

Discussion of table 3. In the comparison of assay results using serum with a known (added) amount of penicillin, the results of table 2 are borne out in practical application. The FDA assay procedure consistently yielded more accurate end points than did the Rosenblatt technique. The explanation again reverts back to the results in table 3, in which it was found that with *Bacillus subtilis* NRRL B-558 a saline-penicillin and a serum-penicillin environment statistically produced a similar end point, whereas with *Streptococcus pyogenes* C-203 a variation in results was encountered. As the calculated result for both methods is dependent upon the standard control set up in a saline-penicillin environment, it is evident that with no variation between the two environments (saline-penicillin and serum-penicillin) the likelihood of obtaining accurate end points is more assured.

DISCUSSION

The foregoing observations suggest that considerable caution must be used in the interpretation of data concerning attainable penicillin blood levels after the administration of various kinds and amounts of penicillin. Discrepancies of results reported by the various groups of investigators perhaps may be reconciled on the basis of lack of uniformity of assay procedures.

It would appear that if *Streptococcus pyogenes* C-203 is to be used as a test organism, the penicillin must be made up in normal human serum. Rammelkamp and Keefer recommend the use of blood broth as the diluent for the standard so that aberrations due to the nutritional effects of serum are avoided. Whereas the Rosenblatt *et al.* determination does not take the serum aberration into account, the Rammelkamp and Keefer technique, involving cumbersome pipetting operations, the use of whole blood, which may cause variation in results, and the high percentage of error obtained in the results, indicates the need for a method which can be easily managed in all routine laboratories. The need is met by the *Bacillus subtilis* procedure recommended by the FDA. Our data indicate that the nutritional effect of serum on the rate of growth of *Streptococcus pyogenes* is sufficient to alter appreciably the results obtained. This phenomenon was not noticed for *Bacillus subtilis*. The nutritional effect of serum on the rate of growth of the test organism has also been commented upon by Kirby and Rantz (1944), but in connection with the turbidimetric assay procedure.

It should also be pointed out that, regardless of method, there is a considerable error involved in any of the recommended assay procedures, and a difference of one tube reading may make a difference of as much as 50 to 100 per cent in the final result.

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THE INFLUENCE OF SUBCULTURE AND OF STREPTOCOCCAL EXTRACTS ON THE GROWTH RATES OF HEMOLYTIC STREPTOCOCCI

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In a previous paper (Lewandowski and Stahly, 1944) it was shown that the growth rate of a group C hemolytic streptococcus, adapted to artificial media, became constant after a number of subcultures in an apparently favorable medium. It is the purpose of this paper to give a description of the materials and methods used and to present the growth rates of a group of strains maintained by passage through mice. The influence of streptococcal extracts on the growth rates of hemolytic streptococci also was investigated and the results are included in this paper.

Relatively few studies concerning the growth rates of hemolytic streptococci have been reported. Mason (1935) calculated generation times for streptococci from a few growth curves which he obtained from the literature. Foster (1921), working with a single strain of hemolytic streptococcus, graphically indicated the change of pH in a standard broth medium during the growth of the organism. Beckwith and Rose (1925) observed that a strain of hemolytic streptococcus, which had been maintained by pleural passage in rabbits and was extremely virulent for these animals, proliferated more rapidly in infusion broth than a strain of the organism which had been maintained in artificial media and had become attenuated. Blundell (1942) suggested that an extract of the washings of a group A streptococcus may act as a growth factor in increasing the virulence of this bacterium for mice.

METHODS AND MATERIALS

Strains employed. The strains of hemolytic streptococci used in this study were stored in the lyophilized condition after repeated passage through mice. Just before use, they were again passed through mice and were then held in blood broth at 7 to 10 C for not more than one week while being studied. These strains are listed, together with pertinent data, in table 1.

Broth medium. The ingredients of the neopeptone broth used were the following:

Difco dehydrated beef heart for infusion	100 g
Difco neopeptone	10 g
Na_2HPO_4 cp	4 g
Double-distilled water	1,000 ml

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To prepare the broth, the beef heart was added to the water and infused at 50 C for 1 hour. The mixture was heated at 80 C for 10 minutes, after which it was filtered through a layer of cheesecloth and then through coarse filter paper. The neopeptone and Na_2HPO_4 were dissolved in the filtrate, and sufficient 1 N NaOH was added to bring the pH to slightly over 8.0. The mixture was boiled for 15 minutes, filtered through paper, and cooled to room temperature. Sufficient distilled water then was added to bring the volume to 1,000 ml, and the pH was adjusted to 7.6. The broth was autoclaved for 20 minutes at 15 pounds, pressure; the final pH was 7.4 to 7.6. This broth supported a scanty growth of the streptococci used in this study.

Sterile horse plasma and sterile glucose were added to the broth immediately prior to inoculation. Enriched with 5.0 per cent plasma or 0.25 to 1.0 per cent glucose, or both, the broth supported a luxuriant growth of the hemolytic streptococci.

TABLE 1

Strains of hemolytic streptococci adapted to mice*

STRAIN	LANCEFIELD GROUP†	GRIFFITH TYPE	MLD FOR MICE
			ml
Griffith B1T	A	2	10^{-8}
Griffith-Franklin	A	5	10^{-8}
Griffith-Symons	A	9	10^{-8}
Griffith-Dochez	A	10	10^{-8}
Griffith NE73T	A	11	10^{-9}
O.S.U.‡	C (human)		10^{-6}

* All strains formed matt colonies on blood agar.

† All group A strains were kindly supplied by Dr. Rebecca C. Lancefield of the Hospital of the Rockefeller Institute for Medical Research, New York.

‡ Isolated in 1936 from a case of scarlet fever at the Ohio State University Hospital. Trehalose-positive and sorbitol-negative (Edwards, 1932).

Determination of growth rates. The photoelectric colorimeter described by Evelyn (1936) was used for measuring the growth. A light filter with a transmission maximum at 660 mμ was used throughout the studies.

In order to obtain the growth curves from which growth rates were calculated, 0.5 ml of a 12-hour broth culture were transferred to an Evelyn tube containing 20.0 ml of broth. The tube was thoroughly shaken and a galvanometer reading taken immediately. Uninoculated broth was used as a control for light transmission. The inoculated tube was placed in a constant temperature water bath at 37 C, and galvanometer readings were made every half hour until maximum turbidity was reached.

The galvanometer readings were converted to photometric densities and the latter then were plotted against time on semilogarithmic graph paper. Curves similar to bacterial growth curves were obtained by this method; these are illustrated in figure 1. It was found that photometric densities bore a linear relationship to the number of organisms in suspension. Growth rates, representing the time in which a twofold increase in turbidity took place, were calculated from the straight line portion of the curve by means of the following formula:

$$g = \frac{t \log 2}{\log b - \log B}$$

where,

g = growth rate
t = time
b = density at the top of the straight line portion of the curve
B = density at the bottom of the straight line portion of the curve.

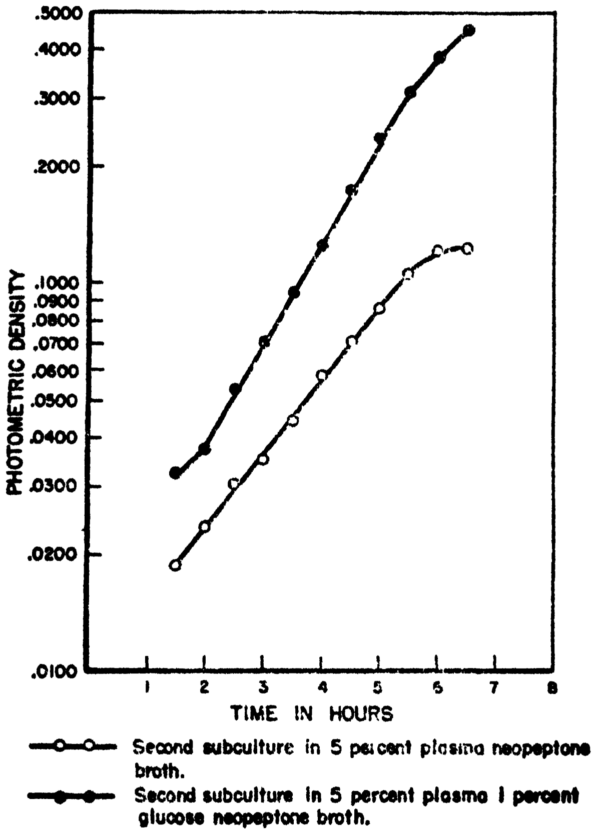

Fig. 1. Growth on the Mouse Passage Griffith NE73T Group A Strain of Hemolytic Streptococci in Various Media

Preparation of streptococcal extracts. Two or three loopfuls of a blood broth stock culture were transferred to 6.0 ml of the plasma glucose broth and incubated for 12 hours at 37 C. A 2.5 per cent inoculum of this culture was introduced into glucose broth and incubated at 37 C either for 3 hours (encapsulated organisms) or 15 hours (unencapsulated organisms).

After incubation, cultures were centrifuged, the supernatant broth was discarded and the sedimented organisms were washed with sterile double-distilled water. The suspensions were again centrifuged, the supernatants discarded, and the sedimented organisms resuspended in sterile double-distilled water in the proportion of 12.0 ml of water for every 100 ml of original broth culture. These suspensions were incubated for 3 hours at 37 C and then centrifuged. The supernatant, after filtration through a Berkefeld N candle filter, contained the sterile extracts used in this study.

Cultures were examined for capsules by means of the India ink method of Butt, Boynge, and Joyce (1936) before, during, and after the extraction process. Capsules disappeared during extraction for 3 hours with distilled water. The extracts had no observable toxic effects when injected intraperitoneally into mice in amounts up to 1.0 ml.

Testing of streptococcal extracts. Inocula of the strains to be tested were prepared by transferring several loopfuls of a blood broth stock culture to 6.0 ml of plasma neopeptone broth. After 12 hours of incubation at 37 C, 0.5-ml amounts of the culture were transferred to an Evelyn tube containing 19.5 ml of plasma neopeptone broth plus 0.5 ml of extract and also to a similar tube containing 20.0 ml of broth only. Growth curves were prepared and growth rates calculated from photometric density data as described above.

TABLE 2

Effect of daily subculture in neopeptone broth on the growth rates of group A hemolytic streptococci in plasma glucose neopeptone broth

STRAIN	GROWTH RATES IN MINUTES	
	Parent culture	Subculture*
Griffith B1T	67	59
Griffith-Franklin	64	53
Griffith-Symons	54	52
Griffith-Dochez	66	62
Griffith NE73T	69	49

* Each of these strains was subcultured 23 times in neopeptone broth before being tested.

RESULTS

Growth rates before and after repeated subculture. The growth rates of five different types of group A hemolytic streptococci were compared in plasma glucose broth before and after 23 daily subcultures in neopeptone broth. Only one of these strains (Griffith NE73T) was highly virulent for mice. All the growth rates are recorded in table 2.

The growth rates of the five strains before subculture ranged from 54 minutes for the Griffith-Symons strain to 69 minutes for the highly virulent Griffith NE73T strain. After 23 daily subcultures in neopeptone broth all strains showed an increase in growth rate in plasma glucose broth. The largest increase in rate of growth (20 minutes) was shown by the virulent Griffith NE73T strain, whereas the smallest increase (2 minutes) was exhibited by the Griffith-Symons strain.

Effect of streptococcal extracts on growth rates. Extracts were prepared of both encapsulated and unencapsulated cultures of the O.S.U. group C parent strain. The effect of these extracts on the growth rates of both the O.S.U. group C and the Griffith NE73T group A parent strains was determined. The results are listed in table 3.

TABLE 3

The effect of streptococcal extracts on the growth rates in plasma neopeptone broth of hemolytic streptococci

STRAIN	GROWTH RATES IN MINUTES		
	PLASMA BROTH	Plasma broth plus extract A*	Plasma broth plus extract B†
O.S.U. group C	87	75	62
Griffith NE73T (group A)	89	74	71

* Prepared from a 3-hour culture of encapsulated organisms of the O.S.U. group C strain.

† Prepared from a 15-hour culture of unencapsulated organisms of the O.S.U. group C strain.

TABLE 4

The effect of streptococcal extracts on the growth rates in plasma neopeptone broth of parent cultures and subcultures of strains of hemolytic streptococci*

MEDIUM	GROWTH RATES IN MINUTES			
	O.S.U. group C		Griffith NE73T	
	Parent	Subculture†	Parent	Subculture‡
Plasma broth	87	64	89	70
Plasma broth plus extract A	79	63	90	79
Plasma broth plus extract B	83	54	85	61
Plasma broth plus extract C	54	70	77	98
Plasma broth plus extract D	110	70	85	58

* Extract A—prepared from the parent O.S.U. group C strain.
Extract B—prepared from the subcultured O.S.U. group C strain.
Extract C—prepared from the parent Griffith NE73T strain.
Extract D—prepared from the subcultured Griffith NE73T strain.

† Twenty-three daily subcultures in neopeptone broth.

‡ Fifty-four daily subcultures in neopeptone broth.

The growth rates of both strains were markedly increased by the extracts. The effect probably was not due to capsular material, however, since the extract prepared from unencapsulated organisms produced a greater increase in the growth rates of both strains than did the extract prepared from encapsulated organisms. The former extract seemed to show some specificity of action, as it increased the growth rate of its homologous O.S.U. group C strain to a greater extent than that of the Griffith NE73T strain.

Since repeated subculture of hemolytic streptococci in neopeptone broth resulted in an increased growth in a favorable medium, it was decided to determine whether subculture also affected the streptococcal extracts. Therefore, extracts were prepared from the O.S.U. group C strain before and after 23 daily subcultures and from the Griffith NE73T strain before and after 54 daily subcultures in neopeptone broth. These extracts were prepared from unencapsulated organisms, i.e., cultures incubated at 37 C for 15 hours. The effect of each extract on the growth rates of both strains, before and after subculture, was studied. The results are recorded in table 4.

In 5 per cent plasma neopeptone broth the O.S.U. group C and Griffith NE73T strains showed an acceleration of growth rate after repeated daily subculturing in neopeptone broth. With one exception, extracts prepared from parent cultures markedly increased the growth rates of parent cultures; the growth rates of the subcultured streptococci were either unaffected or decreased. Extracts prepared from subcultured bacteria increased, with one exception, the growth rates of the subcultured organisms, but the growth rates of parent cultures were either slightly increased or decelerated.

DISCUSSION

The growth rates in an apparently favorable medium of five group A strains of hemolytic streptococci of different types varied from 54 to 69 minutes. All five strains were adapted to mice and were variably virulent for these animals. The strain showing the highest virulence for mice had the slowest growth rate.

After 23 daily subcultures in a poor medium each of the five strains showed an acceleration of growth rate in a favorable medium. The acceleration ranged from 2 minutes to 20 minutes. The strain showing the highest virulence for mice showed the largest increase in growth rate and the fastest final rate. It seems probable that this virulent strain was not able to adapt itself quickly to growth in artificial media but after subculture it became more efficiently adapted than the other less virulent strains. It should be noted that these results do not agree with those reported by Beckwith and Rose (1925).

That hemolytic streptococci possess physiologically active materials is supported by the observation that crude water extracts, prepared either from encapsulated or unencapsulated organisms and added to suitable media, accelerated the growth rates of the organisms from which they were prepared. The acceleration effect of these extracts was not entirely specific; an extract prepared from a group C streptococcus accelerated the growth rate of a group A streptococcus and vice versa. On the other hand, extracts prepared from parent cultures markedly accelerated the growth rates of parent cultures but either had little effect on or markedly decelerated the growth rates of subcultures of the same or different strains. Similarly, extracts prepared from subcultures markedly accelerated the growth rates of subcultures but either had little or a marked inhibitory effect on the growth rates of parent strains. The effects of the extracts were apparently not related either to the serological group of the streptococci studied or to the presence of capsules. It appeared that the effects were related to the physiological state of the cultures from which the extracts were prepared. Apparently, as the streptococci were subcultured, the physiologically active materials changed in character in a manner to aid the growth of the streptococci during the adaptation process.

It is possible that purification and concentration of the streptococcal extracts used in this study would result in greater and more specific acceleration and deceleration effects on the growth rates of hemolytic streptococci. Study of the antigenic and chemical composition of these extracts, as well as their effect on the virulence of hemolytic streptococci, is indicated.

SUMMARY

The growth rates, as measured by the time required for doubling the photometric density in a highly favorable medium, of five group A hemolytic streptococci, adapted to mice, ranged from 54 to 69 minutes. After 23 subcultures in a poor medium each of the five strains showed an increased growth rate in a favorable medium. The acceleration ranged from 2 to 20 minutes.

Streptococcal extracts were prepared which accelerated the growth rates of homologous, as well as heterologous, strains. In general, extracts prepared from parent cultures markedly accelerated the growth rates of parent cultures, whereas extracts prepared from subcultures markedly accelerated the growth rates of subcultures.

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SIMPLE MEDIUM FOR MAINTENANCE OF MENINGOCOCCI

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The smaller Army laboratories such as those in station hospitals and many of the general hospitals are not adequately equipped to identify bacteria fully, and it was therefore required, and was the practice, to send all cultures suspected of being meningococci to some control laboratory for identification and to the Army Medical School for verification. The medium generally employed for the transmission of such cultures was blood agar. A large proportion (in some instances over 50 per cent) of the strains submitted for verification were not viable on arrival at the laboratory.

The problem was considered of sufficient interest and significance to warrant some study to ascertain whether a medium more suitable than blood agar for maintenance of meningococci (and particularly one which could be prepared in the field without the use of special peptones or other nutritive constituents) could be prepared. A medium the base of which consisted of beef extract broth to which was added 1.0 per cent corn starch, 0.01 per cent $CaCl_2$, 0.02 per cent KCl, 0.03 per cent glucose, 0.002 per cent phenol red indicator, and 1.5 per cent or 2 per cent agar, the reaction being adjusted to pH 7.4, was found to be suitable for growth, shipment, and maintenance of meningococci for long periods of time (5 to 8 weeks).

In table 1 it will be noted that of six freshly isolated strains observed, three survived for 3 days and all were dead after 4 days on the routine blood agar, whereas on the blood-free medium, three strains were alive after 12 days and one after 19 days, when storage was in the icebox at a temperature of 5 to 8 C. When stored at room temperature (25 to 28 C), none of the strains were viable after 4 days on blood agar, whereas all were alive after 31 days on the other medium. At 37 C all the six strains had died off on blood agar after 3 days but remained viable on the beef extract, starch, salt, glucose agar after 31 days. Cultures of meningococci grown on this medium were found to be satisfactory as antigens for agglutination reaction, and gonococci grew well after primary isolation.

Reducing the agar concentration to 0.1 or 0.2 per cent and substituting for the 0.03 per cent glucose 0.5 per cent to 1 per cent of a desired carbohydrate yields a very satisfactory fermentation medium for differential diagnosis of meningococci, gonococci, and other varieties of *Neisseria*.

The media for determination of fermentation characteristics, preservation, and shipment of *Neisseria* (particularly meningococci) follow:

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A. Meningitis carbohydrate media (for determination of fermentation reactions of *Neisseria*).

Beef extract broth	1,000 ml
Agar	2 g
KCl (1%)	20 ml
$CaCl_2$ (1%)	10 ml
Starch (corn)	10 g

(Note: The usual sugars are glucose, lactose, maltose, and sucrose.)

TABLE 1

Effect of medium and temperature on survival of meningococci

STORAGE (DAYS)		1	2	3	4	6	12	19	26	31
Temp.	Medium	Number of strains surviving								
5 to 8 C	B. A.	6	6	3	0	0	0	0	0	0
	S. A.	6	6	6	6	6	3	1	0	0
25 to 28 C	B. A.	6	6	3	0	0	0	0	0	0
	S. A.	6	6	6	6	6	6	6	6	6
37 C	B. A.	6	5	0	0	0	0	0	0	0
	S. A.	6	6	6	6	6	6	6	6	6

B. A., blood agar.
S. A., starch, salts, glucose agar.

To prepare 100 ml proceed as follows:

1. To 100 ml beef extract broth add
   a. KCl (1 per cent) .... 2 ml
   b. $CaCl_2$ (1 per cent) .... 1 ml
2. Prepare a starch paste by adding to 1 g of corn starch, in a mortar, 5 to 10 ml of cold water, grinding mixture into a fine paste.
3. Add this cold starch paste slowly to about 50 ml of the medium, boiling continually and stirring. Continue boiling for 5 to 10 minutes. Make up loss with boiling water.
4. Add this 50-ml starch-BXB mixture to the remaining 50 ml of the medium, continually boiling and stirring.
5. Adjust the reaction pH 7.4 to 7.6.
6. Add 0.2 g of agar and boil to dissolve.
7. Make up any loss due to evaporation.
8. Add 10 ml of 0.02 per cent phenol red.
9. To each 100 ml of the medium add 0.5 (or 1.0) g of a desired carbohydrate.
10. Distribute 4- to 5-ml quantities into Loeffler tubes.
11. Sterilize in the autoclave at 10 to 15 pounds for 10 minutes.
12. Remove from the autoclave and cool quickly by placing tubes immediately in cool water.

B. Meningitis stock medium (particularly for preserving meningococci and other varieties of *Neisseria*).

This is prepared in the same manner as described above for "meningitis carbohydrate media" except that glucose only is employed in a concentration of 0.03 per cent (0.3 g per 1,000 ml).

This is distributed in 6- to 8-ml quantities in 16-mm tubes. Sterilization is carried out as described above.

C. Meningitis agar medium (for shipping or preservation of cultures). Same as B but containing 1.5 to 2 per cent agar and prepared as slants.

MELIBIOSE BROTH FOR CLASSIFYING YEASTS[1]

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In her monograph on ascosporogenous yeasts, Stelling-Dekker (1931) used fermentation of various sugars as the principal criterion for separating species of the important genus *Saccharomyces*. Most of the species of industrial importance fall into two species with several varieties in each species. *Saccharomyces carlsbergensis* ferments all the sugar in raffinose broth and *Saccharomyces cerevisiae*, including the important variety *ellipsoideus*, ferments only one-third of this sugar. The separation of these species is of some economic importance since most wine, distillery, and baking yeasts, as well as those used in the brewing of ale, are top yeasts, and those used for the manufacture of beer are bottom yeasts. Most top yeasts are *S. cerevisiae* and most bottom yeasts are *S. carlsbergensis*. This commercial distinction of ale from beer is a convenient one, but it does not seem to be general. Thus *ale* and *beer* in British nations, *Bier* in Germany and in Czechoslovakia where German was understood, *öl* in Scandinavian countries, and *cerveza* in Mexico are words used in these beer-drinking countries more or less commonly for both types of brews. *S. cerevisiae* and *S. carlsbergensis* are closely related, and hybrids between the two are readily produced (Winge and Laustsen, 1939; Lindegren, Spiegelman, and Lindegren, 1944). Although the complete fermentation character is dominant over the one-third fermentation, neither Mrak and his associates (1940, 1942) nor the present authors in hundreds of isolations from nature have encountered more than a very few strains which fermented more than one-third of the raffinose.

Raffinose is a trisaccharide which on hydrolysis yields a fermentable monosaccharide fructose and a disaccharide melibiose. Those strains which ferment only one-third of the raffinose lack the enzyme that splits the melibiose into fermentable sugars, or they have a genetic character which prevents the enzyme from functioning (Lindegren *et al.*, 1944). In his review on yeasts Henrici (1941) suggested that by the use of both raffinose and melibiose one can eliminate the quantitative determination of the residual raffinose. Actually, early workers on yeasts had used melibiose fermentation to separate bottom from top yeasts, and very recent workers as well have used it. Unfortunately, melibiose is costly. At present prices it costs more than gold, gram for gram—about ten times the cost of raffinose. This prevents melibiose from being widely used except for special purposes. The present report shows how a melibiose broth can be prepared from a raffinose broth.

Wickerham (1943) has suggested an ingenious method whereby a separate melibiose broth and a quantitative determination of residual raffinose may both

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be dispensed with, and in the hands of the authors this method has been successfully used. The method necessitates a long period of time, however, as Wickerham noted. He inoculated tubes of raffinose broth with his unknown yeast and allowed the fermentation to proceed until the gas in the tube began to recede, indicating that the CO_2 was being absorbed and no more was being produced—in other words, that no sugar fermentable by the unknown yeast remained. Then the tube was reinoculated, this time with a known melibiose fermenter, e.g., *S. carlsbergensis*. Now if all the raffinose had been fermented by the unknown yeast, the gas continued to recede; but if two-thirds remained—that is, the melibiose portion of the raffinose molecule—the tube again filled with gas. Thus with one tube one can find whether all or only one-third of the raffinose is fermented; or, stated in other terms, whether raffinose and melibiose, or raffinose only, is fermented by the unknown yeast.

Our method was to prepare in liter amounts the following medium: H_2O, 1 L; raffinose, 30 g; and peptone, 10 g. The flask after sterilization was inoculated with a pure culture of *S. cerevisiae* var. *ellipsoideus*. After a week of fermentation at room temperature, which during the course of the experiments varied from 21 to 35 C, usually about 25 to 27 C, the fermentation had ceased. The medium was then dispensed into deep Durham fermentation tubes and resterilized. For convenience we shall here refer to this medium as R melibiose broth. Similar tubes of raffinose broth (without the preliminary fermentation by a yeast) were prepared, and several yeasts were inoculated into each medium. The following yeasts were used. We give names only to those which had been accurately identified. Number 1 is *Saccharomyces globosus*, 2 and 3 are *Saccharomyces cerevisiae*, 4, 5, and 6 are *Saccharomyces cerevisiae*, var. *ellipsoideus*, 13 is *Cryptococcus fermentans*, 14 is *Cryptococcus dactyliferus*,[2] 15 and 16 are

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[2] The most prominent genus of an ascosporogenous yeast is known under three different names: *Torula*, *Cryptococcus*, and *Torulopsis*. It is now generally agreed that *Torula* properly refers to a dematiaceous mold, not to a yeast. Although most medical mycologists have long used *Cryptococcus*, of late years most industrial workers in yeasts have adopted the generic name *Torulopsis*. We retain the name *Cryptococcus* as the older term, as one in wide current usage (Swartz, 1943; Mackie, Hunter, and Worth, 1945; Moore, 1944; Strong, 1944; Cornell, 1944; Conant, Martin, Smith, Baker, and Calloway, 1944; Ash and Spitz, 1945; Skinner, Emmons, and Tsuchiya, 1947), and one that, if it is used at all, can only be used for these yeasts. We believe that it is the valid name (Benham, 1935; Dodge, 1935). We do not believe that *Torulopsis* can be used under the International Rules of Botanical Nomenclature, nor do we believe that it can be validated for the organisms in question as a *nomen conservandum*. If used at all, conserved or not, we believe that it will have to be used to replace *Candida* (where it actually becomes a later homonym of *Syringospora* [Conant, 1940]), as the logic but not the conclusions of Lodder (1934) and Diddens and Lodder (1942) seem to demand, or to replace *Rhodotorula* as Dodge (1935) very clearly maintains. The type species of *Torulopsis* is a red yeast, and the type species cannot be excluded from the genus. Hence the new combinations.

These two species have been described since 1935 without a Latin diagnosis. To retain the specific names and to prevent the creation of other names which those who adhere to International Rules would be compelled to accept, we redescribe them with Dr. Mrak's permission and we give full credit to the original authors as the original authorities for the names. When bacteriologists name new species of *Eumycetes*, they would help prevent con-

Saccharomyces carlsbergensis, 18 is Wickerham's no. 236, 19 his no. 562, 20 his no. 636, and 21 his no. 804.

We knew that no. 1 ferments glucose but not raffinose or melibiose. Therefore no gas should be produced in either medium unless there was some hydrolysis of sugar during sterilization. Likewise we knew that cultures nos. 2 to 14 ferment only one-third of the raffinose. These should form gas in the raffinose, but not in the R melibiose, broth. Cultures 15 to 21 were known to ferment all the raffinose. These should form gas in both raffinose and R melibiose broths. Table 1 shows that our expectations were in all cases realized. *S. cerevisiae* var. *ellipsoideus* removed all the fructose from the raffinose molecule, but it left the melibiose portion; and no demonstrable hydrolysis of sugar took place during sterilization. We use the word fermentation here as Stelling-Dekker used it, to mean gas (CO_2) production under anaerobic conditions.

The second experiment was in part a repetition of the first. The size of the inoculum was controlled after the first experiment, and all media from the same culture had an inoculum of the same size. This was accomplished by removing a loopful of the growth from a 24-hour glucose peptone agar slant, mixing it in 10 ml of sterile water, and using 0.2 ml of this suspension for an inoculum. The number of inoculated cells was thereby cut down to 2 per cent (approximate) of the number previously used. This smaller inoculum, together with the fact that the prevailing temperature during the second experiment was 6 C or more lower, possibly explains the slightly longer time for fermentation to take place. This time we also performed Wickerham's test on all raffinose broth tests by inoculating an active culture of *S. carlsbergensis* into raffinose broth tubes as soon

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fusion in nomenclature if they would add a short Latin description of their new species. Wisely or unwisely, the last International Botanical Congress deliberately chose to retain the rule and to strengthen it. No longer does the rule merely state that a Latin diagnosis must accompany each new name. Now it states that new names of plants other than bacteria, published since 1935 without this diagnosis, are to be disregarded.

Torulopsis fermentans Mrak et McClung. Cellulae sunt aut globosae aut ellipsoideae in culturis maltatis liquidis, juvenes (usque ad 24 horas) 3.5–5.75 × 4.4–8.75, seniores (3 diebus) 3.5–7 × 4.5–8.75 μ. Ascosporae non videtae sunt. Dextrosum, laevulosum, mannosum, galactosum, maltosum, raffinosum (⅓), sucrosumque fermentari possunt neque autem lactosum. Peptonum et spiritus frumenti absorberi possunt, neque autem asparagin, nec urea, nec sulfas ammoniae, nec nitrates. Gelatinum non liquefitur. Habitat in culturis ex baccis *Vitis viniferae* in California. Descriptio extensior lingua anglica est in J. Bact., **40**, 401, 1940. *Cryptococcus fermentans* (Mrak et McClung) Skinner et Bouthilet, comb. nov. Syn: *Torulopsis fermentans* Mrak et McClung.

Torulopsis dactylifera Mrak, Pfaff, et Vaughn. Cellulae sunt aut globosae aut ellipsoidéae aut piriformes in culturis maltatis liquidis, juvenes (usque ad 24 hours) 1.5–4.5 × 2.0–5.3μ, seniores (3 diebus) 2.5–5.2 × 3.0–5.4 μ. Nec pseudomycelium nec ascosporae videtae sunt. Dextrosum, laevulosum, maltosum, raffinosum (⅓), sucrosumque fermentari possunt neque autem galactosum, nec maltosum, nec lactosum. Asparagin, sulfas ammoniae, urea, peptonumque absorberi possunt neque autem nitrates, nec spiritus frumenti. Habitat in culturis ex fructibus fermentantibus *Phoenicis dactyliferae* in California. Descriptio extensior lingua anglica est in J. Bact., **43**, 695, 1942. *Cryptococcus dactyliferus* (Mrak, Pfaff, et Vaughn) Skinner et Bouthilet, comb. nov. Syn: *Torulopsis dactylifera* Mrak, Pfaff, et Vaughn.

TABLE 1

Time for gas to be produced in raffinose broth and in melibiose broth prepared by fermentation from raffinose broth

YEAST CULTURE	RAFFINOSE BROTH	MELIBIOSE BROTH
	days	*days*
1	No gas	No gas
2	1	No gas
3	1	No gas
4	1	No gas
5	1	No gas
6	1	No gas
7	1	No gas
8	1	No gas
9	1	No gas
10	8	No gas
11	1	No gas
12	1	No gas
13	2	No gas
14	2	No gas
15	1	1
16	1	1
17	1	4
18	1	8
19	1	4
20	1	3
21	1	8

TABLE 2

Time for production and recession of gas in raffinose broth, for production of gas after reinoculation with S. carlsbergensis in raffinose broth, and for production of gas in melibiose broth prepared from raffinose

YEAST CULTURE	GAS PRODUCTION, MELIBIOSE BROTH	GAS PRODUCTION, RAFFINOSE BROTH	RECESSION OF GAS, RAFFINOSE BROTH	GAS PRODUCTION AFTER REINOCULATION
	days	*days*	*days*	*days*
1	No gas	No gas		
2	No gas	3	10	5
3	No gas	1	13	7
4	No gas	1	10	2
5	No gas	1	10	3
6	No gas	2	11	5
7	No gas	3	11	5
8	No gas	3	10	5
9	No gas	1	10	5
10	No gas	12		
11	No gas	2	11	3
12	No gas	2	11	5
15	7	1	16	No gas
16	2	1	14	No gas
17	2	1	14	No gas
18	7	2	12	No gas
19	15	2	14	No gas
20	4	1	14	No gas
21	11	2	8	No gas

as gas had definitely started to recede. It will be noted from table 2 that, although the time for gas to be formed was greater than in table 1, those cultures which were slow in table 1 were also the slowest in table 2. Moreover, it will be seen that Wickerham's method is reliable, but slightly slower than our method. If one were to use his method with any of the melibiose-positive cultures, one one would have to add 7 days to the 8 to 16 days that it took for the gas to recede in order to be certain that more gas was not going to be produced by the action of the reinoculated melibiose-positive culture (see last column of table 2).

Although there seems to be some advantage in our method over Wickerham's in the time necessary for making the test, still the advantage is not very great and possibly would not compensate for the extra expense and the trouble of using a double set of tubes. A chance observation, however, showed how the time could be cut down by several days. Ordinarily, all media were inoculated within 48 hours from the time they were prepared. By chance, some tubes of R melibiose broth which had stood around the laboratory until the liquid had evaporated to about one-half volume were inoculated with a culture of a slow melibiose fermenter. Gas was formed promptly, and therefore the possibility of hastening the test by the use of a more concentrated medium was investigated.

TABLE 3

Time for gas production in melibiose broth prepared from pure melibiose, in single and double strength melibiose broth prepared from raffinose by fermentation, and in raffinose broth for recession of gas and for formation of gas after reinoculation with a melibiose-fermenting yeast in raffinose broth

YEAST CULTURE	GAS PRODUCTION PURE MELIBIOSE	GAS PRODUCTION MELIBIOSE PREPARED FROM RAFFINOSE	GAS PRODUCTION, DOUBLE STRENGTH MELIBIOSE BROTH PREPARED FROM RAFFINOSE	GAS PRODUCTION, RAFFINOSE BROTH	RECESSION OF GAS, RAFFINOSE BROTH	GAS PRODUCTION AFTER REINOCULATION
		days	*days*	*days*	*days*	*days*
1	No gas	No gas	No gas	No gas		
3	No gas	No gas	No gas	3	9	10
4	No gas	No gas	No gas	3	8	7
6	No gas	No gas	No gas	3	13	18
16	4	2	2	2	8	No gas
17	4	2	2	2	10	No gas
18	41	9	3	3	13	No gas
19	35	Contaminated	4	3	12	No gas
20	35	7	7	3	17	No gas
21	41	17	5	3	17	No gas

It is well known that the ordinary 0.5 or 1 per cent sugar broths of bacteriological laboratories are unsuitable for yeasts. Gas formation is often very much delayed and may be entirely lacking. Two, three, four, or five per cent sugar is usually used. We had settled on 3 per cent raffinose broth for making our R melibiose broth since it was feared that with larger amounts there might be some residual fermentable sugar due to alcohol accumulation. With 3 per cent raffinose there would be only 1 per cent sugar fermentable by *S. cerevisiae* and varieties, and 2 per cent (approximate) residual melibiose. We evaporated a liter of some freshly prepared R melibiose broth by boiling *in vacuo* to one-half volume, to make an approximate 4 per cent melibiose broth. This is called double strength R melibiose broth in tables 3 and 4.

We also used for comparison a broth made with 3 per cent pure melibiose (Difco brand). Table 3 demonstrates that pure melibiose is much slower to ferment than the melibiose in our R melibiose broth. The reason may possibly be found in the fact that the R melibiose broth is a medium enriched by growth-promoting substances from the previous growth in it of yeasts. Table 3 also shows that gas formation in double strength R melibiose broth is more rapidly initiated than in single strength broth. Also the table demonstrates that the concentrating of R melibiose broth to half-volume did not hydrolize the sugar since no gas was produced by a glucose-fermenting yeast.

To verify that the double strength R melibiose broth was actually quicker to initiate fermentation than the single strength broth, we repeated this part of the experiment by inoculating melibiose fermenters in single and double strength R melibiose broths. Table 4 shows again that the delay in the fermentation of the double strength broth is no more than 8 days. In comparison with the long delay in Wickerham's method for gas to recede and for more gas to be produced on reinoculation and with the not quite so long a time necessary in using single strength R melibiose broth, this is of considerable advantage for routine work.

TABLE 4

Time for gas production in single and double strength melibiose broth prepared from raffinose

YEAST CULTURE	GAS PRODUCTION, SINGLE STRENGTH MELIBIOSE BROTH	GAS PRODUCTION, DOUBLE STRENGTH MELIBIOSE BROTH
	days	*days*
1	No gas	No gas
3	No gas	No gas
15	2	1
16	2	2
18	9	4
19	12	3
20	7	7
21	10	5

SUMMARY

Wickerham's technique for the separation of melibiose-fermenting and non-melibiose-fermenting yeasts is accurate, but it is slow.

By fermenting away the fructose portion of raffinose in 3 per cent raffinose broth with *Saccharomyces cerevisiae* var. *ellipsoideus*, one has a medium with melibiose as the only sugar. This fermented medium may be resterilized and used as a melibiose broth. Such a medium will give a slightly more rapid differentiation of melibiose-fermenting from non-melibiose-fermenting yeasts than will Wickerham's method.

If 3 per cent raffinose broth is fermented by *S. cerevisiae* var. *ellipsoideus* and the melibiose broth remaining is concentrated to half-volume and resterilized in fermentation tubes, one can use these to obtain a differentiation of melibiose and non-melibiose fermenters in 7 or 8 days, in contrast to the 3 weeks or more necessary with Wickerham's method, or the 2 or 3 weeks necessary when using the unconcentrated melibiose broth prepared from raffinose.

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THE IDENTIFICATION OF SPOREFORMING BACTERIA ISOLATED FROM INCOMPLETELY STERILIZED AGAR[1]

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Townsend and Zuch (1943) found that heating culture media in bottles for 30 minutes at 115.6 C did not completely sterilize the media. They isolated 23 cultures of aerobic sporeforming bacteria from heated samples of 4 lots of agar and 5 cultures from contaminated vaccines and media, but made no attempt to identify them. Twenty-four were listed as facultative thermophiles with an optimum temperature of 37 C, and 4 as mesophiles.

Since a study of the aerobic mesophilic sporeformers (Smith, Gordon, and Clark, 1946) had shown that some of them were able to grow at fairly high temperatures, it appeared desirable to survey the group isolated by Townsend and Zuch to determine how many cultures belonged to the mesophilic group.

IDENTIFICATION OF CONTAMINANTS

The descriptions of the various species identified and the methods used may be found in the publication of Smith, Gordon, and Clark (1946) and need not be repeated here. After a study had been made of each of the 28 isolates, the following identifications were made:

(1) *Bacillus subtilis*, 14 cultures (50 per cent of the total). The cultures, labeled C, D, 11, 12, 14, 15, 16, 17, 19, 20, 21, 23, 24, and 26, show less variation in their characters than might be expected in this number of isolates. Perhaps the restricted source of the cultures (heated agar) was responsible for this. They all grew at 54 C (water bath) but failed at 56. They are all gram-positive.

(2) *Bacillus pumilus*, 5 cultures (18 per cent of the total). The cultures, numbered 1, 2, 3, 4, and 5, were identical as far as could be determined. Growth occurred at 48 C (water bath) but failed at 50.

Since about 1900 there has been much confusion concerning the identities of this species and *Bacillus mesentericus*. Chester (1903) and later Lawrence and Ford (1916) stated that *B. pumilus* was identical with *B. mesentericus*, the latter being retained as the species name. Unfortunately, the European strains of *B. mesentericus* are not the same as the American. They ferment starch and produce nitrites from nitrates, whereas the American strains do neither. Gibson (1944) has rightly placed *B. mesentericus* as a synonym of *B. subtilis*. Smith,

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[1] This study was made possible by the co-operation of C. T. Townsend, University of California, and T. L. Zuch, National Canners Association, San Francisco, California. They supplied transplants of their isolates and other data without which this work would not have been possible.

Gordon, and Clark (1946) have agreed with Gibson and have recommended the retention of the name *B. pumilus* and the dropping of the name *B. mesentericus* because of the ambiguity. The latter name, therefore, becomes *nomen dubium.*

(3) *Bacillus megatherium*, 4 cultures (14 per cent of total). Numbers 9, 10, 18, and 22 are very similar to one another but vary in some minor respects from typical strains of this species. In the first test arabinose and xylose were not fermented and scant growth occurred on glucose nitrate agar, as contrasted with normal cultures which are positive in all three respects. The cultures under study might, therefore, be considered as weakened or aberrant strains. In that case it should be possible to rejuvenate them. That happened in the case of no. 18. After a few transfers and aging in a casein digest broth, growth was very abundant on glucose nitrate agar and acid was formed from arabinose and xylose. Although the three others did not respond to this treatment, it is not thought that there is really any fundamental difference between no. 18 and the others. They are, therefore, considered as weakened cultures. The maximum temperature allowing growth was 48 C (water bath).

(4) *Bacillus firmus*, 1 culture (3.5 per cent of total). Number 8 is a typical strain of this species. Since *B. firmus* does not seem to be well known, it might be well to state briefly its outstanding characteristics. Morphologically it resembles *B. subtilis* or *B. pumilus*, although frequently the sporangia may be bulged somewhat. Physiologically it fails to grow under acid conditions, a pH of 6.0 or below being inhibitive. No growth, therefore, occurs on potato. Growth on glucose nutrient agar is inhibited because of the slight acid produced. Ammonium salts and urea are not usually used as sources of nitrogen. Number 8 grew at 43 C but not at 45 (water bath).

(5) *Bacillus lentus*, 1 culture (3.5 per cent of total). Number 13 varies slightly from the normal in that gelatin is attacked somewhat and nitrates are reduced to nitrites. *B. lentus* is very similar to *B. firmus*, differing from it in the production of urease and the inability to hydrolyze casein. Number 13 grew at 43 C (water bath) but failed at 45.

(6) *Bacillus circulans*, 1 culture (3.5 per cent of total). Culture no. "B" is slightly aberrant for this species. It fails to attack gelatin at all, whereas normal strains do, although sometimes weakly. Carbohydrates are not attacked so vigorously as usual, the pH of glucose nutrient broth after 7 days' incubation being neutral or slightly alkaline, whereas it should be well below pH 6.0. These differences are not considered important, especially in the case of this species the strains of which show a great variation in the character of the growth and in physiology. Maximum water bath temperature allowing growth was 52 C.

(7) *Bacillus brevis*, 1 culture (3.5 per cent of total). Culture "A" is a typical representative of the species. The maximum temperature allowing growth of various strains of this species varies greatly (Smith, Gordon, and Clark, 1946). Culture "A" grew, although inhibited, at 54 C, this placing it among those strains of the species that grow at the higher temperatures.

(8) Not identified, 1 culture (3.5 per cent of total). Culture "E" is a facultative thermophile, if one may use such a term. It grows very slowly and weakly

at 28 C, best at 50, is inhibited at 57, and does not grow at 58 (water bath). It prefers an alkaline reaction and will not grow at pH 6.0. It seems to be identical with certain so-called thermophiles the classification of which will have to wait for further information on the thermophilic group.

DISCUSSION

It is of more than passing interest that none of the 28 isolates were true thermophiles and that all but one could be classified as belonging to established mesophilic species. Of special note is the fact that *Bacillus subtilis* accounted for half of the cultures, whereas *Bacillus cereus* was not found among them. The latter is the most numerous sporeformer in soil and frequently occurs elsewhere (Smith, Gordon, and Clark, 1946). It is not known whether its absence here was due to the low heat resistance of its spores or to other, perhaps ecological, reasons.

In studying cultures incubated at temperatures above 37 C, it must be borne in mind that there is a difference between air and water bath temperatures, the higher the temperature, the greater the differential. In one set of observations using a small electrically heated incubator, an air temperature of 50 C gave a water bath temperature of about 46. When the air temperature was raised to 56 C, the water was about 50, and if it was raised to 62, the water was about 54 C. Whether these ratios of air to water temperatures would hold for other incubators is not known. It is nessessary, therefore, to observe the temperature of the water during every test. The use of air temperature may lead to quite serious discrepancies, as one can easily see. For instance, *B. subtilis* is listed as having growth limits of 10 C to 56 C (Bergey *et al.*, 1939). Smith, Gordon, and Clark (1946) found considerable variation among different strains of this species in the maximum temperature at which growth occurs. Usually the limit was between 50 and 54 C (water bath). One, however, failed to grow above 37, whereas several grew at 58, although inhibited somewhat. If air temperature had been recorded, the upper limit would have been about 65 C and might have led one to believe that he had a thermophile.

Another reason for using a water bath for high temperatures is to avoid the lag in temperature that occurs when the cultures are incubated in the air. Growth of these rapidly developing bacteria may occur during the period of temperature adjustment and give a false impression. To avoid this, the tubes of media used in this work were brought up to the required temperature in a water bath, quickly inoculated, and placed in the incubator containing another water bath maintained at the required temperature. There was, therefore, no lag in the temperature of the culture and results could be duplicated.

SUMMARY

A study of 28 cultures of sporeforming bacteria previously isolated by Townsend and Zuch from contaminated agar and vaccines resulted in the following identifications: *Bacillus subtilis*, 14 cultures; *B. pumilus*, 5; *B. megatherium*, 4; *B. firmus*, 1; *B. lentus*, 1; *B. circulans*, 1; and *B. brevis*, 1. One unidentified

culture belongs to a group intermediate between the mesophiles and the thermophiles, a group that has not been described adequately.

The absence of true thermophiles and the repeated occurrence of *B. subtilis* among these cultures are especially noteworthy.

For determining the maximum temperatures of growth, it is recommended that cultures be incubated in a water bath for temperatures above 37 C and that the medium be brought up to the same temperature before inoculation.

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ELECTRON MICROSCOPE STUDIES OF THE "INTERFERENCE PHENOMENON" BETWEEN BACTERIAL VIRUSES OF THE ESCHERICHIA COLI GROUP

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The interference phenomenon between different bacterial viruses attacking the same host was demonstrated by Delbrück and Luria (1942). They showed that mixed infection of a bacterium with Tα and Tγ resulted in suppression of the growth of Tα and that Tγ reproduced normally. Interference with Tα by Tγ occurs even when Tα is added as long as 4 minutes before Tγ. The authors suggested that interference is due to competition for a "key enzyme" present in limited amounts in each bacterial cell. In a later paper Luria and Delbrück (1942) showed that γ particles inactivated by ultraviolet radiation retain their interfering ability and that a single γ-inactivated particle per bacterium is sufficient to suppress the growth of Tα. The interfering ability, although more resistant to ultraviolet than the reproducing ability, is eventually destroyed by larger doses of radiation. The irradiated virus also inhibits the growth of the bacterium by which it is destroyed.

Interference is known to occur in the plant and animal virus groups. (See Delbrück and Luria, 1942, for a short review.)

The following work was suggested by Dr. Delbrück and conducted by the authors, using the electron microscope at the University of Illinois. It consists of visual examination of mixed infection under high magnifications in order to add direct visual evidence of the phenomenon and to gain any possible further information as to mechanisms.

The plating techniques and the one-step growth techniques are those described by Delbrück in the 1942 review. The terminology of Tα as T_1 and Tγ as T_2 and the data on the physical characteristics of viruses are given by Delbrück (1946).

MATERIALS AND METHODS

The bacterial viruses, T_1 and T_2, which are the same viruses as Tα and Tγ of Delbrück and Luria, were used. The same B (*Escherichia coli*) strain served as the host as in their experiments. Difco nutrient broth plus 0.5 per cent NaCl was used as the liquid medium. Titers were determined by the plaque assay method described by Ellis and Delbrück (1939).

For the electron microscope sample preparation, the desired amounts of each of the viruses were added to 2½ hour B cultures, which were continuously aerated at 37 C. Adsorption was allowed to occur, and samples were taken from the tubes at definite times. They were placed on the collodion membrane by a capillary pipette. The collodion membrane, prepared in the usual manner and supported by a fine mesh wire screen (Marton, 1941), was held between clamped forceps. The forceps holding the samples were placed in a moist chamber and allowed to remain there 5 to 10 minutes. After this time, the screens were washed in the meniscus of distilled water and allowed to dry in air. No temperature control was attempted in the moist chamber. The type-B R.C.A. electron microscope was used at 60,000 k.v.

RESULTS

The morphology of T_1 and T_2, as revealed by the electron microscope, has been adequately described by Luria and Anderson (1942), and by Luria, Delbrück, and Anderson (1943). The photographs obtained in this work agree very well with those published. T_1 has a spherical head about 50 mμ in diameter. The tail, however, is visible only occasionally and never appears to be clearly in focus. The head is uniformly dense. T_2 has an oval head in which can be seen granules. The granules visible in the accompanying figures take on the variety of shapes described by the above-mentioned authors and explained by them as areas of different thicknesses. The tail of T_2 is clearly seen in all photographs. The important point to be mentioned here is that T_1 and T_2 show completely different morphology and that it is easily possible, in the same preparation, to distinguish between the two types in the electron micrographs. The results are illustrated in the following typical experiment:

Experiment 12

	Amount mixed	*After clearing*	*Virus/B*
B count 3 hr	2 8 × 10^8/ml		
T_1	5 6 × 10^8/ml	4 × 10^8/ml	2
T_2	1.4 × 10^9/ml	2 6 × 10^9/ml	4

T_1 and T_2 were added simultaneously.

In figure 1, no. 1, which was taken from experiment 12, both T_1 and T_2 are observed on the same cell in approximately the numbers added. These preparations were dried before the end of the latent period of T_1 to prevent adsorption of virus released at that time. Figure 1, no. 2, shows cells which have been lysed on the collodion membrane. The sample was placed on the screen at 20 minutes and was dried at 30 minutes. No T_1 is observed in any of the lysed cells. There is no increase in the titer of T_1 over the original inoculum as shown by the assay method. Many T_2 are observed on the membrane. By the time the sample is completed, about 75 per cent of the cells are destroyed and each shows many T_2. Assays by the plaque method indicate only a slight increase in the amount of T_2. This will be discussed later.

In another experiment higher ratios of virus to bacteria were used as follows:

Experiment 13

	Amount added	*After clearing*	*Virus/B*
B count 2¼ hr	1 × 10^8/ml		
T_1	1 24 × 10^9/ml	1.0 × 10^9/ml	12
T_2	1.5 × 10^9/ml	2.3 × 10^9/ml	15

T_1 and T_2 were added simultaneously.

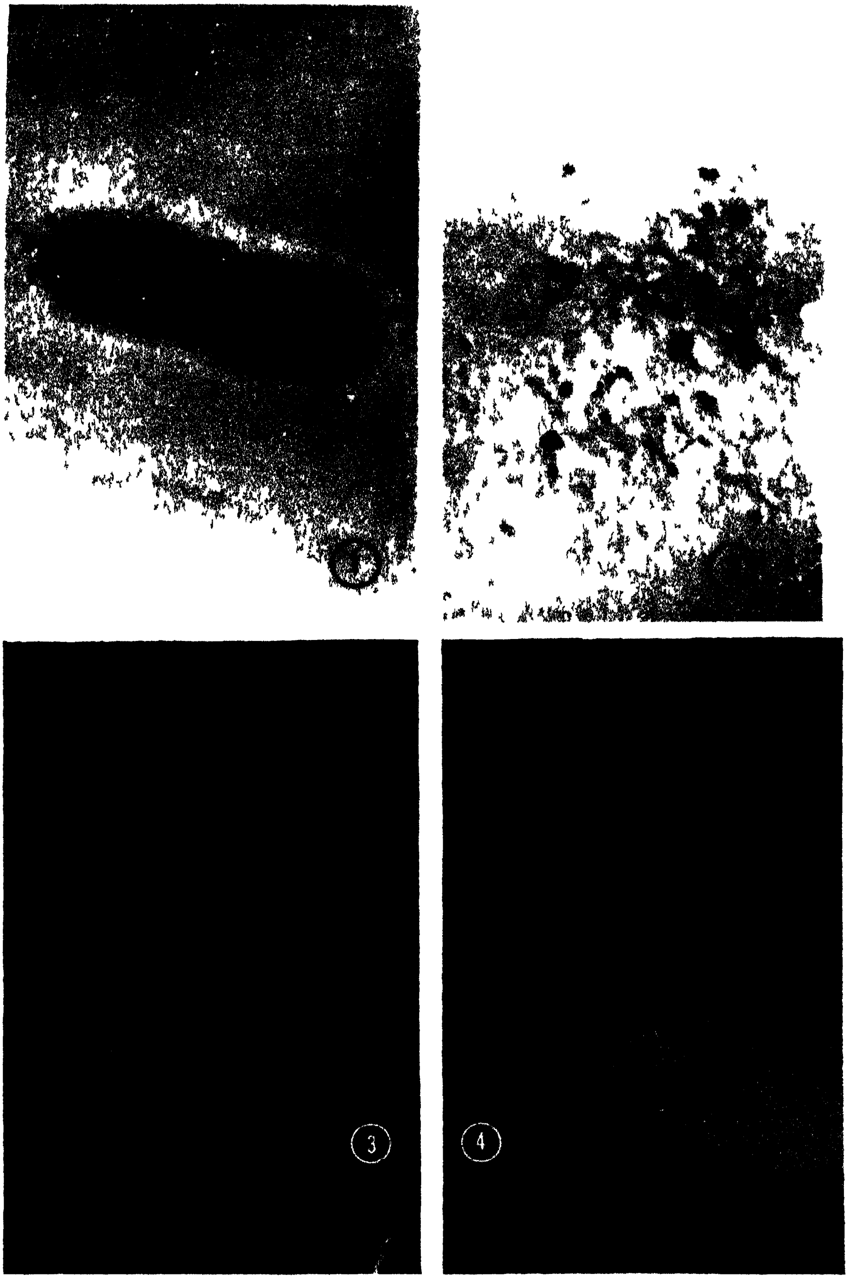


FIGURE 1

No 1 B T_1 and T_2 10 minute contact, showing adsorption of both T_1 and T_2. (ca 23,000 ×)

No 2 B both viruses 20 minute contact showing only T_2 in lysed cell (ca 23 000 ×)

No 3 B—both viruses, showing adsorption of large quantities of T_2 on B (ca 23 000 ×)

No 4 B both viruses, 30 minute contact, dividing cell shows adsorption of both T_1 and T_2 (ca 23,000 ×)

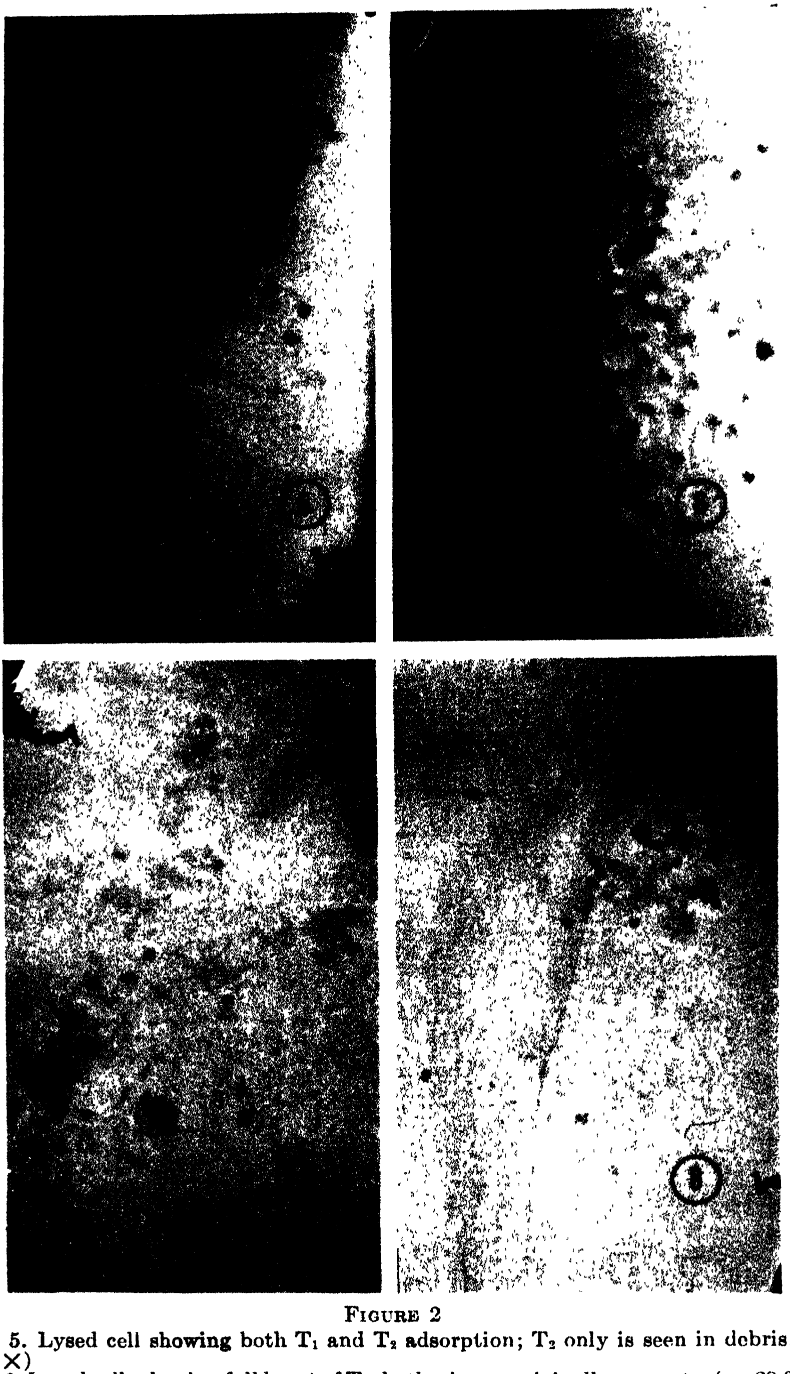


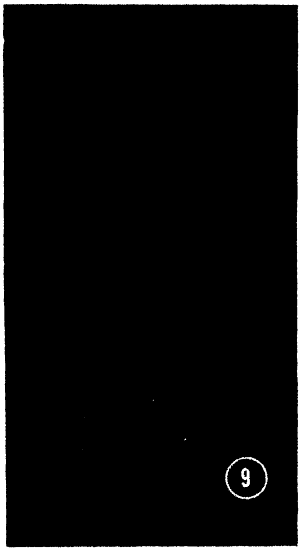
FIGURE 2

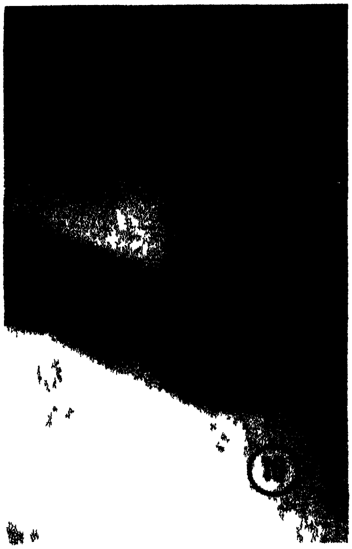
No. 5. Lysed cell showing both T_1 and T_2 adsorption; T_2 only is seen in debris. (ca. 28,000 X)

No. 6. Lysed cell releasing full burst of T_2; both viruses originally present. (ca. 28,000 X)

No. 7. "Incomplete"-appearing T_2 in debris of lysed cell. (ca. 28,000 X)

No. 8. Clumped T_2 from sample after clearing. (ca. 28,000 X)





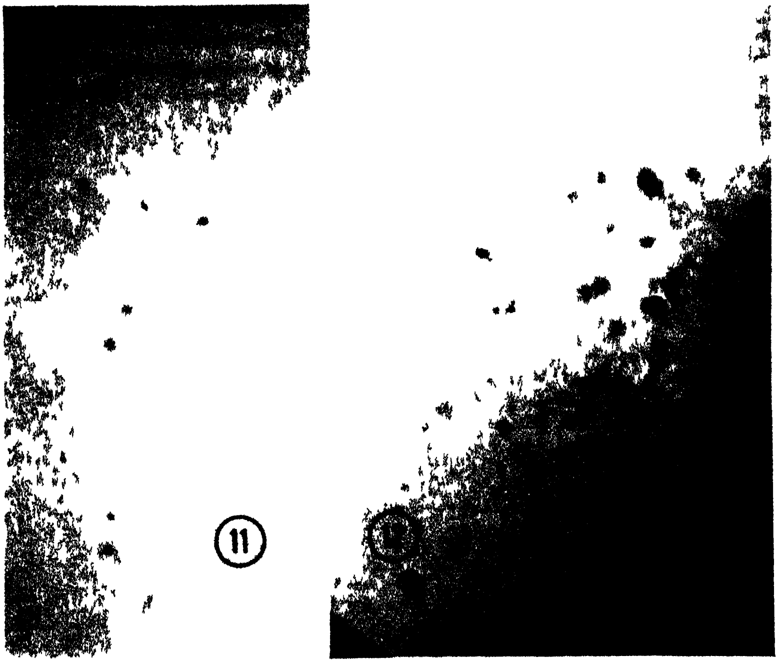


FIGURE 3

No 9 Clumped T_2 in filtered T stock (ca 28 000 ×)

No 10 Cell showing adsorption of both types of viruses Experiment 10 (ca 28 000 ×)

No 11 Sample dried 30 minutes after mixing B with both viruses, showing only T_1 particles (ca 38,000 ×)

No 12 Sample completed 30 minutes after mixing B and T , fixed with osmic acid fumes (ca 28,000 ×)

The samples prepared before lysis in experiment 13 were not sufficiently clear for reproduction, although observation revealed adsorption of both types of virus. The samples prepared (nos. 3 and 4, figure 1) during the "rise period," showed extreme amounts of adsorption of T_2 and some adsorption of T_1. This is to be expected since T_2 is being released and is adsorbing on adjacent cells. The entire cell surface is covered by the virus. As previously pointed out by Delbrück, Luria, and Anderson, adsorption appears to be at random in relation to the head and tail of the virus. In no. 4, viruses may be seen adsorbed in the region of constriction of this dividing cell. Both T_1 and T_2 can be seen. In the lysed cells of these same samples large quantities of T_2 may be seen in the cell debris (figure 2, nos. 5, 6, and 7). In no. 5, T_1 as well as T_2 are visibly adsorbed to the surface of the destroyed cell membrane.

The titer of T_1 has not increased by any detectable amount. In both experiments it is slightly lower than the inoculum, although the reduction is probably not significant. The titer of T_2 has increased by less than a factor of 2, although large quantities of T_2 are present in the cell debris of the lysed bacteria. Figure 2, no. 6, shows over 100 particles of T_2, as do other photographs of the lysed cells. Some of these particles may have been those adsorbed. In no. 8, T_2 particles clumped and adsorbed to bits of cell debris are seen. Figure 3, no. 9, shows the same phenomenon in a filtered and bacteria-free stock.

In experiment 10, 1.2×10^8 per ml T_1 were added to a bacterial culture containing less than 1×10^8 per ml B several minutes before T_2 (2×10^8 per ml) was added. Both T_1 and T_2 can be observed on the cell surface in the samples dried before the end of the latent period of T_1 (figure 3, no. 10). However, in the debris of cells lysed on the membrane during sample preparation only T_1 is observed (no. 11). Unfortunately no final titers were assayed since this was a preliminary experiment. It is cited, however, because, although both viruses are observed adsorbed to the membrane of the bacteria, only one type of virus appears to be released.

Many other photographs of other experiments prepared in this work verify these results.

DISCUSSION

From these photographs, as well as from the data of other investigators, it is evident that both T_1 and T_2, when mixed in equal quantities and added simultaneously to the host, are adsorbed by the host. It is also evident that a host cell yields an almost normal burst of only one of the two viruses and that the reproduction of the other is either completely or greatly inhibited.

The electron micrographs can be taken only in support of other experiments, since there is always the possibility either that T_1 is present in small quantities, being obscured by a greater amount of the larger virus, or that it has been removed by the washing process. It is very likely that many virus particles are removed at this time; however, a cell which has burst on the membrane and has the virus still entangled in its debris shows a full burst. Many experiments with

T_1 indicate that T_1 remains to a large extent on the membrane through the washing process.

The titers of T_2 obtained in this laboratory were always lower than expected. They never exceeded a titer of over 5 × 10^9, and the burst size, as determined from one-step growth curves of either single or multiple infection, was always in the order of 20 to 30. The electron micrographs indicate a burst size of over 100, which agrees with the published figures of Delbrück. The figures obtained from electron micrographs, of course, cannot be taken too seriously, since many of the particles present are adsorbed. Figure 2, no. 8, shows clumping of the T_2 particles in the lysate of single cells, whereas no. 9 shows clumping in the filtered stock. Photographs of stocks of T_1 and T_5 do not show similar aggregations. The particles are distributed singly and evenly over the membrane surface. It is suggested that the T_2 particles remain clumped and thus reduce the titers of T_2, since each aggregate would act effectively as one virus particle. The cause of the clumping in this laboratory is unknown, since the factors of broth type, salt concentration, and pH used by other investigators were reproduced as closely as possible.

In the electron micrographs a peculiar "incomplete" appearance of the T_2 particles in the lysate of a single cell is seen (no. 7). The head granules sometimes appear to be present in the absence of the remainder of the head material and the tail. This may be an artifice introduced by the drying process. They maintain this appearance, however, even where the osmic acid fixation has been applied (no. 12). It is certainly possible that the head granules are laid down first in the virus synthesis.

SUMMARY

Host *Escherichia coli* infected with both T_1 and T_2 was examined under the electron microscope. Studies of the electron micrographs show that both viruses are adsorbed on the same cells but that only one type of virus appears in large quantities later in the cell debris. This offers visual confirmation of the interference phenomenon demonstrated by Delbrück and Luria.

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THE ASSAY OF ANTIBIOTIC MIXTURES

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As most antibiotics exhibit maximum activity only against a limited number of pathogenic species, it is obvious that the control of certain mixed infections can be most advantageously accomplished by the use of appropriate antibiotic mixtures. Prerequisites to the formulation of such mixtures are assay methods for accurate determination of each component, singly and in combination. Such assays are especially required for stability studies and the determination of possible interactions.

Heilman (1945) has utilized the slide culture technique for the assay of penicillin-streptomycin mixtures. The sensitivity of the organisms, a hemolytic streptococcus and a strain of *Bacillus megatherium*, permitted the assay of each component in the presence of a considerable excess of the other. However, the discontinuity of the results obtainable by 2-fold serial dilution methods precludes the use of such assays when accuracy is essential.

The following studies were undertaken in an effort to devise plate assays suitable for the determination of single components in the following mixtures: penicillin-streptomycin, gliotoxin-streptomycin, and gliotoxin-penicillin.

METHODS

General assay procedure. The assays of the three component antibiotics were made on nutrient agar by the filter paper disc method.

The method for gliotoxin and streptomycin is similar to that described in the Food and Drug Administration's minimum specifications for streptomycin (November 20, 1945). The primary layer consisted of 20 ml of agar adjusted to pH 8.0 to 8.2 before sterilization. The secondary layer of 3 ml was adjusted to pH 8.0 immediately before inoculation, with 0.5 per cent of a spore susepension (7.0×10^7 spores per ml) of *Bacillus subtilis*, A.T.C.C. 6633. After the discs were placed 0.1 ml of the appropriate dilution was added to each disc within 3 seconds. The plates were incubated at 28 to 30 C for 16 to 20 hours.

The penicillin assay was conducted similarly on pH 7.0 nutrient agar. Inoculation was with 0.5 per cent of a 24-hour culture of *Staphylococcus aureus* FDA 209p. Incubation of the plates was at 37 C for 16 to 20 hours.

After incubation the zones of inhibition were determined in the usual manner, and the quantities of each antibiotic were calculated by reference to a daily standard curve plotted on semilog paper. The buffer solutions employed in the assays were 0.05 M phosphate.

Assay of gliotoxin in gliotoxin-streptomycin mixtures. The sample was diluted to approximately 50 units of gliotoxin per ml with pH 7.0 buffer containing 10 per cent by volume of 0.01 M potassium periodate (Van Dolah and Christenson, 1946). After this stood 1 hour at room temperature, further dilutions were made with pH 8.0 buffer. The assay range employed for gliotoxin was 3 to 25 μg per ml. The treatment with potassium periodate destroys the streptomycin but the gliotoxin is unaffected.

Assay of streptomycin in gliotoxin-streptomycin mixtures. The sample was diluted with pH 8.0 buffer to contain approximately 3 μg of streptomycin per ml. An equal part of chloroform was added, and the mixture was shaken vigorously. After separation of the layers, an aliquot of the aqueous phase was further diluted to give approximately 3, 2, 1, and 0.5 μg per ml. This extraction removes the gliotoxin, but the streptomycin is quantitatively retained in the water layer.

Assay of gliotoxin in gliotoxin-penicillin mixtures. The sample was diluted to approximately 40 to 50 μg per ml of gliotoxin with pH 7.0 buffer. The penicillin was then inactivated by the addition of a 10 per cent volume of a penicillinase[1] solution containing 200 penicillinase units per ml. After this stood 1 hour, further dilutions were prepared in pH 8.0 buffer.

TABLE 1

Assay of known mixtures

ASSAY OF	OTHER COMPONENT	PROPORTION	RECOVERY	NO OF PLATES
			%	
Gliotoxin	Streptomycin	1:1	99	24
Streptomycin	Gliotoxin	1:1	99	24
Gliotoxin	Penicillin	1:1	101	8
Penicillin	Gliotoxin	1:1	100	8
Streptomycin	Penicillin	1:1	98	24
Streptomycin	Penicillin	10:1	100	22
Penicillin	Streptomycin	1:1	100	16
Penicillin	Streptomycin	10:1	100	16

Assay of penicillin in penicillin-gliotoxin mixtures. If the gliotoxin to penicillin ratio was 1 or less, the sample was assayed directly with *Staphylococcus aureus*, since this test organism is relatively resistant to gliotoxin. In the presence of larger quantities of gliotoxin, chloroform extraction of a buffered solution of the mixture is advisable.

Assay of streptomycin in streptomycin-penicillin mixtures. The sample was diluted in pH 7.0 buffer to approximately 50 μg per ml of streptomycin, and 10 per cent of a penicillinase solution containing 200 units per ml was added. After 1 hour further dilutions were prepared in pH 8.0 buffer.

Assay of penicillin in streptomycin-penicillin mixtures. Penicillin was assayed in the presence of as much as 10 parts of streptomycin, by using *Staphylococcus aureus* FDA 209p. The usual assay range was 3 to 0.3 μg per ml.

Various known inactivators of streptomycin were employed in an effort to find a selective agent. All gave some destruction of penicillin.

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[1] A generous sample of penicillinase was supplied by the Schenley Laboratories.

RESULTS

A series of known mixtures was assayed by the foregoing techniques. The results were read from standard curves prepared from the single components and are reported as percentages of recovery. The number of plates that were employed to obtain each average value are reported in the right-hand column of table 1.

A limited stability study has been made on a penicillin-streptomycin mixture. The determination of streptomycin in this study was made by the 8-plate double-dose method described in the Food and Drug Administration's specifications. Control assays were performed on the single components, lyophilized in amounts identical with those in the mixture. The results are presented in table 2. Each figure represents the average value obtained by the assay of duplicate vials.

This study was not of sufficient duration to ascertain definitely the stability of streptomycin at either temperature. A small decrease in potency can be noticed, but this lies within the assay error. As would be expected, the penicillin assay decreased substantially at room temperature.

TABLE 2

Stability study of a penicillin-streptomycin mixture

	SINGLE COMPONENTS		MIXTURE	
	Penicillin U/vial	Streptomycin μg/vial	Penicillin U/vial	Streptomycin μg/vial
Initial assay	9,900	72,000	9,400	68,000
2 weeks at 4 C.	9,800	68,000	9,400	61,000
2 weeks at R.T.*	9,100	69,000	9,100	66,000
4 weeks at 4 C.	8,300	69,000	8,300	58,000
4 weeks at R.T.	7,400	55,000†	8,100	60,000
8 weeks at 4 C.	8,700	67,000	9,200	62,000
8 weeks at R.T.	6,900	61,000	7,600	59,000

* R.T. designates storage at room temperature (26 to 28 C).

† The assays on the duplicate vials did not check. One had a content of 42,000 μg, the other 67,000 μg.

DISCUSSION

The assay methods described are adaptable to mixtures containing widely different proportions of the antibiotics with the exception of the method for the determination of penicillin in the presence of streptomycin. Here, the proportions assayable depend on the sensitivity of the strain of *Staphylococcus aureus* employed. Unfortunately, the known inactivators of streptomycin are not sufficiently specific for use in the presence of the unstable penicillin molecule.

The assay of ternary mixtures was not attempted, but with some limitations the procedures described should be adequate for such combinations.

ACKNOWLEDGMENT

The authors are indebted to Mr. L. Nisonger and his assay group for the streptomycin determinations reported in table 2.

SUMMARY

Methods are described for the assay of penicillin, gliotoxin, and streptomycin in binary mixtures of these antibiotics.

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THE INACTIVATION OF INFLUENZA VIRUS BY CERTAIN VAPORS

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Although numerous studies have been reported on the virucidal activity of various substances used as aerosolic mists or in direct contact with the influenza virus, only two studies have been published describing the action of vapors on viruses, so far as the writers are aware. Remlinger and Bailly (1942) showed that when the viruses of rabies, pseudorabies, or eastern equine encephalomyelitis were exposed to the vapors of eucalyptus oil in a static atmosphere at 21 C, these viruses were innocuous when subsequently injected into susceptible animals. Exposure to the vapors for 5½ hours to 2 days and 8 hours, the time depending upon the virus used, was required to destroy infectivity. Recently Stone and Burnet (1945) found that vapors of bromine, chlorine, and especially iodine inactivated the influenza virus. When mice were placed in a chamber containing atomized influenza virus and sufficient iodine vapor, the mice did not become infected. This protection was shown to result from inactivation of the virus in the air rather than from a prophylactic or therapeutic action of the vapors on the mice.

The present report describes a simple *in vitro* method for detecting the virucidal action of vapors from volatile substances and gives the results obtained with 48 substances tested by this method. A brief account is also presented of the treatment of infected mice with vapors of one of the substances that inactivated the influenza virus *in vitro.*

EXPERIMENTAL

In vitro studies. With the exception of a few tests with the Lee strain of the influenza B virus, to be noted later, the PR8 strain of the influenza A virus,[1] which had been passed numerous times through mice and fertile eggs, was employed in all experiments.

Pools of infected allantoic fluids having an average chicken cell agglutination (CCA) titer of 1:1,024 were stored in dry ice until required for an experiment, at which time the fluids in lusteroid tubes were liquefied by immersion in tepid water. The fluids were then diluted to 10^{-2} in an acetate buffer of pH 7.0 and chilled in the refrigerator at 5 C for a brief period before exposure to the vapors under test.

The essential elements of the apparatus employed for testing the virucidal action of vapors are shown in figure 1.

Air(1) from a pump is passed through a glass tube(2) containing absorbent cotton (to remove airborne bacteria) into a larger glass cylinder(4) in which a gauze wick impregnated with 0.5 ml of the volatile substance is placed. The

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[1] We are indebted to Dr. Frank L. Horsfall, Jr., for the original virus material.

vapor-containing air then passes down a glass tube(7) and bubbles through 2 ml of diluted virus suspension(10) to an outlet(11), which is connected by rubber tubing to a flowmeter. Various volatile substances and dilutions of virus suspension can be quickly and easily tested by changing the tubes containing the wick(4) and diluted virus suspensions(10). To facilitate impregnation of the gauze wick, many of the substances were diluted or dissolved in triethylene glycol, since this solvent, when utilized in this manner, did not inhibit growth of the virus. Two of the compounds, as indicated in table 1, were melted and placed on the wick, since a suitable solvent was not found. Although different concentrations of the various test substances were used, this is unimportant in the interpretation of the results because the vapor pressure of a volatile substance remains constant through a considerable range of concentrations.

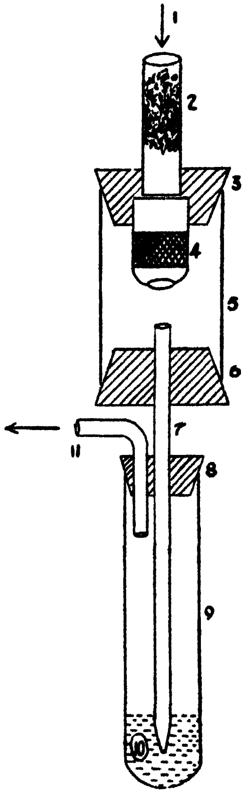

FIGURE 1

1. Air inlet.
2. Tube with absorbent cotton.
3. One-hole rubber stopper.
4. Impregnated gauze wick.
5. Glass cylinder.
6. One-hole rubber stopper.
7. Glass tube.
8. Two-hole rubber stopper.
9. Large test tube.
10. Diluted virus.
11. Air outlet.

In all experiments conducted with this apparatus the air flow was arbitrarily adjusted to 15 ml per second and bubbled through the diluted virus suspension at room temperature for 10 minutes at this rate. Immediately after exposure to the vapors, 0.1 ml of the treated virus was inoculated intra-allantoically into 10- or 11-day fertile eggs, which were incubated at 37 C for 48 hours. Following incubation, the eggs were chilled overnight at 5 C to prevent bleeding, and the undiluted allantoic fluid from each egg was tested for its CCA activity.

On the first trial of a compound each of 10 eggs was inoculated with 0.1 ml of the vapor-treated virus. If the compound exhibited inhibition of CCA activity in the allantoic fluids of several of the eggs, the test was repeated one or more times. In the preliminary work it was observed that the bubbling of air alone through a 10^{-2} dilution of the virus for 10 minutes at room temperature caused a reduction in the infectivity of the virus. A control 10^{-2} dilution, held at room temperature for 10 minutes without the bubbling of air through it, showed no reduction in infectivity. If, however, the 10^{-2} dilution was first chilled at 5 C, no detectable destruction of the virus occurred when air alone (at room temperature) was bubbled through the virus for 10 minutes. When 10^{-3} or 10^{-4} dilutions of the chilled virus were used, inconsistent results in the air controls were obtained; and hence all tests reported in this paper were made with the 10^{-2} virus dilution. Nevertheless, to detect any significant inactivation of the virus caused by the air itself, an air control was included in each day's tests.

Table 1 indicates the compounds that have been tested and their action on the influenza A virus. It will be noted that the compounds which consistently produced 100 per cent inactivation of the virus were α-naphthyl isocyanate, β-naphthyl isocyanate, phenyl isocyanate, and *p*-nitrobenzoyl chloride. Oxyquinoline, thiourea, oil of nutmeg, and oil of mustard showed only a slight degree of inhibition.

It is important to note here that the lack of CCA activity in the first egg passage does not necessarily constitute unequivocal evidence that the virus has been destroyed by some experimental procedure, for Ziegler, Lavin, and Horsfall (1944) demonstrated that when irradiated influenza virus was injected into fertile eggs, allantoic fluids from these eggs failed to display CCA activity, but when these fluids were inoculated into a second set of eggs, fluids from this second passage showed full CCA activity. Thus CCA activity may be removed without completely destroying the infectivity of the virus. In order to determine whether or not vapor treatment of the virus destroyed infectivity as well as CCA activity, the following test was performed: Virus dilutions were exposed to the four most effective vapors by the methods previously described and were inoculated into eggs which were incubated 48 hours. One-tenth ml of the allantoic fluid from each of these eggs was then inoculated into a second set of eggs, which were in turn incubated 48 hours. When a CCA test was performed on the fluids from the second egg passage, no agglutination occurred, indicating that the vapors had inactivated the infectivity as well as the hemagglutination of the virus. Further passages were not carried out since Ziegler, Lavin, and Horsfall (1944) have demonstrated that when active virus was not detected in the second passage it did not appear in the third passage.

TABLE 1

Action of vapors from volatile compounds on the influenza virus

COMPOUND	CONCENTRATION	SOLVENT	NO. EGGS TESTED	EGGS SHOWING POS. CCA	COMPOUND	CONCENTRATION	SOLVENT	NO. EGGS TESTED	EGGS SHOWING POS. CCA
	per cent			*per cent*		*per cent*			*per cent*
α-Naphthyl isocyanate	100		45	0	*p*-Thiocresol	5	T.G.	10	100
β-Naphthyl isocyanate	100*		25	0	Thioglycolic acid	80	H_2O	10	100
Phenyl isocyanate	100		25	0	Thiosemicarbazid	5	T.G.	10	100
Phenyl isothiocyanate	100		20	100	Cyano acetic acid	10	T.G.	10	100
p-Nitrobenzoyl chloride	100*		35	0	Oleic acid	100		10	100
Methyl benzoyl acrylate	5	T.G.†	10	90	Salicylaldehyde	20	T.G.	20	100
Oil of cloves	100		10	100	Triethylene glycol	100		10	100
Oil of eucalyptus	100		10	100	Methyl alcohol	100		10	100
Oil of nutmeg	100		10	90	Ethyl alcohol	100		10	100
Oil of mustard	20	T.G.	10	90	*n*-Propyl alcohol	100		10	100
Turpentine	100		10	100	Isobutyl alcohol	100		10	100
Thymol	50	T.G.	20	100	Sec. butyl alcohol	100		10	100
Thymol sulfonic acid	5	T.G.	10	100	Tert. butyl alcohol	100		10	100
Chlorothymol	50	T.G.	20	95	*n*-Amyl alcohol	100		10	100
Menthol	50	T.G.	10	100	Isoamyl alcohol	100		10	100
Oxyquinoline	20	T.G.	10	80	Sec. butyl carbinol	100		10	100
Methyl cinnamate	50	T.G.	20	100	Diethyl carbinol	100		10	100
Diethyl maleate	5	T.G.	10	100	*n*-Hexyl alcohol	100		10	100
As. di. *n*-butyl urea	5	T.G.	10	100	*n*-Heptyl alcohol	100		10	100
Thiourea	5	T.G.	10	70	*n*-Decyl alcohol	100		10	100
Allyl thiourea	5	T.G.	10	100	*n*-Undecyl alcohol	100		10	100
Phenyl urea	5	T.G.	10	100	Lauryl alcohol	100		10	100
Octyl isourea	5	T.G.	10	100	Myristyl alcohol	5	T.G.	10	100
Thiophenol	5	T.G.	10	100	Cetyl alcohol	5	T.G.	10	100

* = melted.

† = triethylene glycol.

In vivo tests. Since the *in vitro* tests were so conclusive in demonstrating the inactivation of the influenza virus by four of the compounds tested, it was considered of interest to determine whether any of these compounds would be therapeutically effective in treating mice infected with the influenza virus. Mice were therefore inoculated intranasally with 0.05 ml of PR8 mouse lung virus diluted 10^{-3} and 10^{-4}. They were then placed in large glass desiccators with openings to permit the entrance and exit of vapors.

Vapor treatment of the mice was effected by bubbling air at the rate of approximately 5 ml per second through undiluted α-naphthyl isocyanate and passing the vapor-laden air simultaneously into desiccators containing infected and uninfected mice. Various treatment periods were used, the maximum being 8-hour periods for 7 days. Uninfected control mice given this treatment exhibited no apparent toxic effects. However, both the infected treated and the infected untreated mice usually died between the fifth and seventh day after inoculation, and the animals killed and autopsied did not reveal any significant difference between the treated and the untreated groups in the extent or degree of lung consolidation. It was therefore concluded that although the vapors of α-naphthyl isocyanate were relatively nontoxic, they were without demonstrable therapeutic effect on mice infected with influenza A virus. The other three compounds shown to be highly virucidal *in vitro* were not tested *in vivo* because a satisfactory solvent was not found for β-naphthyl isocyanate and *p*-nitrobenzoyl chloride, and phenyl isocyanate was a strong lachrymator.

DISCUSSION

On the basis of our first studies we adopted the hypothesis that compounds which were known to react with amino acids might also prove virucidal. It may therefore be significant that, of the 48 compounds tested, the four that were most effective in destroying the virus have a common characteristic of reacting readily with amino acids. It is obvious, however, from our results, that there are compounds which react with amino acids but do not inactivate the influenza virus. Tenbroeck and Herriott (1946) also found that reagents known to react with amino, tyrosine phenol, or SH groups of proteins did not, in some cases, completely inactivate the viruses tested. These workers implied that the well-known virucidal action of formaldehyde is primarily due to its reactivity with amino groups of proteins, though it also attacks other radicals. They reported that mustard $(Cl—CH_2—CH_2)_2—S$, which reacts with proteins, inactivated several viruses without destroying their antigenic value as vaccines. It is possible that some of the virucidal compounds described in this paper might be used for a similar purpose.

Foter (1940) reported that allyl, methyl, and ethyl isothiocyanate produced bactericidal vapors for a number of organisms, but oil of mustard (allyl isothiocyanate) and phenyl isothiocyanate vapors failed to inactivate the influenza virus in the present work.

The inability of α-naphthyl isocyanate vapors to protect infected mice might be attributed to a number of factors such as lack of absorption, inactivation by the tissues, insufficient concentration, etc. Stone and Burnet (1945) were also unable to protect infected mice with iodine vapors although these vapors were highly effective in destroying the virus in the air.

A few tests were conducted to determine the action of α-naphthyl isocyanate and β-naphthyl isocyanate vapors on the Lee strain of influenza B virus. It appeared that this virus was just as susceptible to these compounds as the PR8 strain.

SUMMARY

A simple *in vitro* method for testing the virucidal action of vapors from volatile substances is described.

The infectivity of influenza A virus was completely destroyed by suitable exposure to the vapors of α-naphthyl isocyanate, β-naphthyl isocyanate, phenyl isocyanate, and *p*-nitrobenzoyl chloride. Oxyquinoline, thiourea, oil of nutmeg, and oil of mustard showed very slight virucidal action. None of the other 40 compounds tested *in vitro* exhibited any appreciable virucidal activity. The influenza B virus was also inactivated by the vapors of α- and β-naphthyl isocyanate.

The vapors of α-naphthyl isocyanate did not protect mice infected with influenza A virus under the conditions of the experiment.

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CONDITIONS CONTRIBUTING TO STREPTOCOCCAL VIRULENCE

II. Intra-Phasic Attenuation by Sulfanilamide

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During recent years many studies have reported the degree of bacteriostatic or germicidal influence of sulfonamide drugs on numerous bacterial species. In some instances these studies have also suggested possible mechanisms whereby these effects were produced. Suggestions have also been made why bacteria, when submitted continuously to the influence of one or another sulfonamide drug, tend to become drug-resistant or "drug-fast," thereby being able to develop well in drug concentrations that earlier inhibited their growth or even killed them outright. The protecting role of *para*-aminobenzoic acid, for example, seems to have been demonstrated in several bacterial species; and the suggestion has been made that other substances produced by bacteria in their normal growth may aid in protecting them against the inimical action of certain drugs. Although these studies originated in the province of disease therapy, they have served to uncover new problems in bacterial physiology and have thus become concerned in some measure with the subject of bacterial variability.

It is natural that in the majority of the earlier studies primary attention should have been paid, for therapeutic reasons at least, to bacteriostatic or germicidal aspects. And, along with this focusing of attention on the elimination of the bacteria from the blood or tissues, or upon the inhibition or destruction of organisms in the culture tube, there have been few instances in which special attention was given to the question of what, if anything, was happening to the morphological attributes, the virulence, or the culture state of the organisms during the period of development of their drug fastness or as a result of continued bacteriostasis *in vitro* or *in vivo*. The question remains as to what influence the drugs may have on virulence as a bacterial attribute distinct from the ability of the organisms to undergo multiplication when in contact with increasing concentrations of the drugs. For, in a general way, it is recognized that virulence and ability to grow well in a culture tube do not necessarily have much in common. This subject is given consideration in the present paper; also the subject of the range and extent of intra-phasic variations in virulence (either attenuation or exaltation) as contrasted with that of inter-phasic variations.

In an earlier paper we (1943) considered the extent to which the primary and potential virulence for mice (MLD, 0.7 ml) of a derived rough-phase alpha streptococcal culture known as the Conzello strain, originally obtained in smooth phase from the blood stream of a hospital patient dying of subacute bacterial endocarditis, could be enhanced by serial mouse passages. It was shown that the first increase in virulence was associated with inter-phasic variation involving the transformation *in vivo* from rough to smooth phase. But it was shown further that the greatest part of the total increase in virulence occurred after the rough form had disappeared and as the passages continued with the smooth phase. The smooth culture observed at the time of maximal virulence (MLD, 0.000001 to 0.0000001 ml) was similar in morphological, colonial, and other cultural respects to the smooth-phase culture as it existed at the time of isolation from the patient, and when it was used to generate the rough form.

Thus, at the end of the series of mouse passages in the earlier experiments, we possessed a culture of exceptional virulence for a smooth-phase strain of alpha hemolytic streptococcus related etiologically to endocarditis lenta. The question therefore arose: Would it be possible to bring about a corresponding decrease in the virulence of this culture by employing some means of continuous contact with the drug (in this case sulfanilamide), and to accomplish this degree of attenuation within the smooth (intra-phasic) range? To do this within an interphasic range (as, for example, the smooth-to-rough transition) would have no point of special interest—aside from its special bearing on the species concerned—in view of the fact that, under the latter conditions, reduction of bacterial virulence has been reported for many species.

The present study therefore had two aims: (1) to ascertain the influence of continued contact of the virulent, smooth culture with a sulfonamide in reducing virulence; (2) to ascertain whether it was possible to bring about such a reduction of virulence without at the same time effecting recognizable dissociative or other changes in the culture.

CULTURE AND METHODS

The culture of greening streptococcus employed was the same as that used in the earlier study.[1] It was known in the laboratory as the "Conzello smooth"

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[1] Because of uncertainties in present-day criteria for ascertaining the taxonomic position of strains of greening and indifferent streptococci, indeed, uncertainties in knowing what actually constitutes a streptococcal "species," we did not attempt, at the time of our earlier paper, to designate the species or even the group represented by the Conzello culture. And we shall do so now only to the extent of presenting some of its cultural, biochemical, and morphological characteristics in respect to matters that have often been used for the attempted differentiation of streptococcal "species" or groups. We wish to do this particularly because, as Rosebury (1944) has commented, the virulence of the Conzello smooth at the time of isolation was considerably higher than that usually regarded as characteristic for strains of greening and indifferent streptococci derived from the subacute form of endocarditis.

On blood agar the culture grew more luxuriantly than do the majority of hemolytic or greening streptococci from human sources with the exception of some members of the enterococcus group. At the time of isolation the smooth colonies gave small zones of greening unaccompanied by hemolysis. Later in the culture's history both smooth and rough colonies on aging showed diffuse and incomplete hemolysis. Hemolysis also appeared in some broth cultures and was tentatively regarded as representing an acid hemolysis. At the present time the smooth colonies, when crowded, give at 24 hours broad zones of diffuse greening and fixation of the blood cells directly under the colonies. Well-isolated colonies give at 48 hours broad, irregular zones of greening and also fixation of cells beneath the colonies. There is no hemolysis. The organisms in smooth phase are elongated diplococci and had been isolated in February, 1942, from the blood stream in a fatal case of subacute bacterial endocarditis. This culture directly after isolation possessed a mouse virulence equivalent to an MLD of 0.0025 to 0.005 ml. From this culture there was produced the rough phase, which had a mouse virulence of 0.7 ml. A new derived smooth, obtained from this rough after a long series of mouse passages, eventually came to possess a virulence of 0.000001 to 0.0000001 ml. This smooth culture was used in the experiments now to be reported.

Dilutions of sulfanilamide were prepared from a 1 per cent solution of this drug in warm proteose peptone broth made from beef infusion and containing 0.2 per cent glucose. The initial pH was 7.4, and 3 per cent of fresh, defibrinated rabbit blood was added when called for. A preliminary test made for the purpose of ascertaining the range of drug dilutions that gave growth inhibition in too strong a degree showed that the organisms grew as well in the 1:80,000 dilution of the drug as in the control tube. Growth in the 1:40,000 dilution at 24 hours was slightly inhibited, and growth in the 1:20,000 and 1:10,000 dilutions was inhibited somewhat more. Eventually, however, a heavy growth occurred in all dilutions. The inoculum for these tubes was one drop (approximately 0.03 ml) of young broth culture, and it is probable that inhibition would have been shown more definitely had the inoculum been less. Since there was sufficient growth of the organisms in the tube of 1:10,000 S.A. dilution at the end of 24 hours, this drug concentration was used for beginning the serial broth passages.

In this dilution 5 passages were made at intervals of about 24 hours. Next there were 5 passages in tubes of 1:5,000 dilution, followed by 6 in 3,000 dilution. Subsequent passages were made in dilutions of 1:2,000, 1:1,000, 1:500, 1:250, and 1:100. Most commonly each S.A. broth tube was inoculated with one drop of the preceding culture in the series. It will become apparent from the results presented subsequently that the length of this series of transfers was greater than was required to obtain significant results.

In order to ascertain whether a similar series of passages in the same medium lacking sulfanilamide would, in itself, determine any significant alteration of virulence or in the cultural or morphological characters of the organism, a series of tubes not containing the drug was conducted separately and with the same intervals and amounts of culture transfer. At certain selected points in both the S.A. and the control series the respective cultures were examined for (1) cell morphology and cell grouping as observed in stained preparations, (2) colonial

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resembling enterococci, although occasional chain formation appears. The latter aspect was naturally intensified in the rough-phase cultures. Serological grouping indicated a member of group G. This result does not conform well with expectations based on the chief characteristics of group G streptococci as presented by Sherman (1937).

Among the sugars fermented were maltose, sucrose, salicin, arabinose (irregularly), and trehalose. Not fermented were lactose, glycerol, mannitol, raffinose, inulin, xylose, and sorbitol. The final reaction in glucose broth was pH 4.6. Ammonia was not produced; gelatin was not liquified; milk was not acidified nor coagulated. There was a faint reduction of methylene blue, and growth was inhibited by 0.1 per cent of this dye. No growth occurred at 10 C or at 45 C. It did not occur in the presence of 6.5 per cent of NaCl or at pH 9.6. The organisms were killed in broth culture at 60 C for 30 minutes.

and other cultural features as indicated by growth on blood agar plates and in broth, and (3) mouse virulence.

Since it did not seem feasible to inject the mice involved in virulence tests with inoculums direct from the serial S.A. broth cultures themselves because of possible unrecognized effects from the drug content, subcultures to plain broth medium without blood were made from selected tubes of both series. From 16-hour growths of these cultures appropriate dilutions in the same medium were prepared, checked for colony count by the pour plate method, and injected at once into mice of uniform size and weight. The same procedure was used for the S.A. broth tubes as for the control broth tubes except in respect to the degree of culture dilution, which became progressively smaller in successive tubes of the S.A. series.

Although an attempt was made to keep as uniform as possible the number of organisms in the inoculums administered to mice from cultures of the two series, this was possible only within fairly wide limits, for it sometimes happened that colony counts failed to reveal accurately the number of organisms in the inoculums. This was largely due to the tendency toward chain formation at times in one or the other series. Under such conditions it was believed that the observation of relative turbidities in broth gave a more reliable index of the number of organisms present in the respective inoculums; and this method of adjustment was accordingly often used. For the most part, however, divergencies in cell count were not sufficiently great to render in any degree uncertain the fact of considerable difference in the results of mouse inoculations from cultures in the respective series.

As shown in detail in the accompanying tabulation (table 1), the tests of virulence were not made from every culture passage but only from cultures of the fourth, seventh, tenth, thirteenth, twentieth, twenty-eighth, and thirty-ninth passages. The series, as it later appeared, was unnecessarily extended with reference to the original purpose in view, since a significant attenuation occurred early in the S.A. series. Passages were maintained, however, even into the 1:100 dilution of the drug, because of the possibility of observing the smooth-to-rough transformation, and therewith a presumably still greater degree of attenuation of culture virulence. This event never occurred.

For each culture tested in each of the two series, three dilutions were used, and, with each dilution, one or two mice were inoculated. This small number of mice seemed adequate for detecting results of significance in view of the long-continued nature of the experiment, which afforded ample opportunity for any false trend to become clear. The range of culture dilutions used for the control mice remained fairly constant since, here, the virulence was well retained over the period of test. In the S.A. broth series, however, the culture concentration of the inoculums increased in relation to the diminishing virulence of the organisms undergoing passage. The actual inoculums in this series ranged from 0.000001 ml to 0.05 ml. The virulence, as tested just previous to the beginning of the experiment, was indicated by an MLD of 0.000001 ml, a slight reduction following the passages being anticipated.

The accompanying tabulation presents in brief form the concentrations of the drug in the S.A. broth dilutions in relation to the passage numbers referred to in table 1.

Passage no. in series	*Concentration of drug*
1 to 5	1:10,000
6 to 10	1:5,000
11 to 16	1:3,000
17 to 18	1:2,000
19 to 20	1:1,000
21 to 22	1:500
23 to 24	1:250
25 to 39	1:100

The final test of virulence was made on the culture of the thirty-ninth passage and after a final 15 consecutive passages in the 1:100 S.A. broth; also, *pari passu*, after 39 passages of the culture of the control series in plain broth. In the fifteenth passage in the S.A. 1:100 dilution the culture grew as well as at any time in its earlier history. And at this point both series were discontinued.

EXPERIMENTAL RESULTS

The chief results from passing the virulent Conzello smooth culture through increasingly strong concentrations of sulfanilamide in broth and from passing the same organism through plain broth (control series) are shown in table 1. The more significant points may be discussed briefly under the following headings:

Virulence. It is shown in table 1 that, beginning with the fourth passage in S.A. broth (S.A. dilution 1:10,000) in which there was observed, at the outset, a slight bacteriostatic effect, the virulence had, at that point, been reduced to such an extent that mice were not killed by inoculums of 0.0001 ml, 0.00001 ml, or 0.000001 ml, while all the mice in the control series of the same passage number died following injection of the control passage culture with the exception of the highest dilution. The mice receiving the indicated dilutions of the culture from the S.A. broth series were not even visibly sick. These results indicate a considerable reduction of virulence even after 4 passages in a concentration of the drug not much greater than that obtainable in the blood stream of treated patients.

After the seventh passage in S.A. broth (involving 5 passages in 1:10,000 and 2 passages in 1:5,000) mice survived injection of 0.01 ml, 0.001 ml, and 0.0001 ml, while the parallel control mice died following injection of 0.0001 ml and 0.00001 ml.

Coincident with still further passages in the more concentrated S.A. broth, the virulence of successive cultures continued to diminish although there was no diminution in volume of growth, and the virulence of the organisms in the control series experienced only slight reduction. In the thirteenth passage culture, for example, the mice in the S.A. series were killed by 0.1 ml but not by 0.05 ml, though 0.00001 ml of the control culture still represented a fatal dose.

From the results of still further passages it appears that the virulence of the S.A. broth culture became so reduced that the MLD for mice tended to become stabilized in the neighborhood of 0.1 ml to 0.2 ml. This was the situation at the

TABLE 1

The gradual attenuation of virulence for mice of the virulent Conzello smooth strain accompanying serial passages in sulfanilamide broth containing increasingly greater concentrations of the drug

SULFANIL. BROTH SERIES*			CONTROL BROTH SERIES*		
Passage 4 (S.A. 1:10,000)					
0.0001 L	0.00001 L	0.000001 L	0.0001 D	0.00001 D	0.000001 L
Passage 7 (S.A. 1:5,000)					
0.01 L	0.001 L	0.0001 L	0.0001 D	0.00001 D	0.000001 L
Passage 10 (S.A. 1:5,000)					
0.1 L	0.05 D	0.02 D	0.0001 D	0.00001 L	0.000001 V
Passage 13 (S.A. 1:3,000)					
0.2 D	0.1 D	0.05 L	0.001 D	0.0001 D	0.00001 D
Passage 20 (S.A. 1:1,000)					
0.3 (a)D (b)D	0.2 (a)D (b)L	0.1 (a)L (b)L	0.001 (a)D (b)D	0.0001 (a)L (b)L	0.00001 (a)D (b)D
Passage 28 (S.A. 1:100)†					
0.3 (a)D (b)D	0.2 (a)D (b)D	0.1 (a)D (b)D	0.001 (a)D (b)D	0.0001 (a)D (b)D	0.00001 (a)D (b)L
Passage 39 (after 15 P in S.A. 1:100)					
0.5 D	0.2‡ D	0.1§ L	0.001 D	0.0001 D	0.00001 L

[*] D = died; L = lived; V = test vitiated.

† At this point in the S.A. series the virulence had dropped far below the level shown by the original Conzello smooth culture at the time of isolation from the patient (MLD, 0.0025 to 0.005 ml).

‡ The apparent incongruity between the results from the twentieth and twenty-eighth passages was probably due in part to the difference in the number of organisms constituting the respective inoculums. As ascertained by plating, the number in the culture of the twenty-eighth passage of the S.A. series was about three times greater than for the culture of the twentieth passage.

§ A mouse receiving 0.05 ml of the same culture also lived.

end of passage 39, following 15 final passages in S.A. broth dilution 1:100, and probably somewhat earlier since the degree of change in virulence during the final passages was very slight. At the end of the series the virulence of the S.A.

culture was considerably below that of the smooth form isolated from the patient. At that time in the culture's history the approximate virulence for mice was 0.005 ml to 0.0025 ml. Thus, over the entire series of 39 passages in S.A. broth the virulence of this smooth-phase culture became reduced from an MLD of approximately 0.00001 ml to 0.2 ml, while the virulence of the plain broth control culture, over the same period and following the same number of passages, became reduced from approximately 0.00001 ml to 0.0001 ml. The greater part of this reduction had occurred at the end of the twentieth passage, though significant attenuation had occurred at the end of the seventh passage.

Culture phase and cell morphology. At the beginning of this study the organisms submitted to serial passage in S.A. broth or plain broth were in characteristic smooth phase. In this phase they remained throughout the passages with the exception of a few brief excursions toward the rough. There were occasional brief periods, extending over 2 or 3 passages, when the smooth colony aspect became slightly modified or when the morphology of the cells underwent slight change, particularly in grouping. These temporary modifications occurred in both series.

In the second and third passage tubes of the S.A. broth series there appeared a considerable number of long chained forms made up of unusually large cocci. The presence of these tended to make plate counts unreliable in their indication of the actual number of cells present. The corresponding colonies presented a slightly matt surface, suggesting the beginning of development of the rough phase. But, with further passage, the organisms recovered from this tendency and the smooth appearance returned.

In another instance, late in the control series, many of the colonies on blood agar plates developed at the colony borders rough "fringes" or "outbursts." This change occurs only when some of the cells in a smooth-phase culture are entering the smooth-to-rough transition. With still further passages this tendency also disappeared, and the unmodified smooth colony form returned.

When the passages had entered the S.A. 1:100 dilution, the first culture in this dilution failed to produce hemolysis (here regarded as an acid hemolysis) in S.A. blood-broth cultures, although the passage strain had done so earlier in the series, and although the control culture continued to manifest such hemolysis.

At the period of its maximum virulence (0.000001 to 0.0000001 ml) observed previous to the beginning of the present experiment, the original smooth culture grew in broth with a homogeneous clouding as contrasted with the flocculent and sedimentary form of growth characteristic of nonvirulent or of slightly virulent cultures. As the passages in S.A. broth progressed, the tendency for the culture to grow as a flocculent sediment at the bottom of the tubes became more marked and continued to the end of the series. This manner of growth in broth, which is observable in some degree in probably the majority of greening or indifferent streptococci isolated from patients experiencing endocarditis lenta, denotes either lack of virulence or weak virulence, whereas cultures of moderate to high virulence almost invariably manifest diffuse clouding. In some cultures from these as well as other sources both forms of growth may appear in the same tube.

These relationships held for the cultures in the two passage series being considered. The organisms in cultures of the S.A. series (losing virulence) showed a much greater flocculating tendency in broth than did cultures in the control series (maintaining virulence). Here, even in the final passages, the broth growth was still fairly homogeneous.

Despite the slight irregularities in cell or colonial morphology noted above, the important point to be observed is that in neither series of passages were there any very significant or long-continued departures from those attributes characterizing streptococcal cultures lying within the smooth range. In particular it may be noted that the long-continued contact with sulfanilamide did not produce a transformation to the rough phase, in which, as was shown by us in an earlier paper (1943), the virulence of this streptococcal culture attained its lowest level. This failure of the smooth culture to attain the rough phase under the influence of sulfanilamide is remarkable when it is recalled how frequently and how easily this transformation can be accomplished in numerous bacterial species by the employment of numerous chemical, as well as nutritional and physical, agents.

Degree of permanence in loss of virulence. It has sometimes been shown that continued contact of a virulent culture with increasing concentrations of a sulfonamide drug brings about a loss of virulence that is only temporary; and that virulence may be regained after a few passages on a favorable, drug-free medium. It has also been indicated, particularly for the pneumococcus, that the so-called "adaptation" of the organisms to a sulfonamide drug is not always accompanied by appreciable loss in virulence; and this conclusion seems to have support from clinical observations.

It has been shown above that the latter situation did not hold for the smooth-phase culture of the Conzello streptococcus. But the possibility that the organisms recovered at the end of the series of passages might easily regain all or a part of their lost virulence when returned to a favorable medium was taken into consideration, and certain tests designed to throw light upon this possibility were performed.

The relatively nonvirulent smooth-phase culture at the end of the thirteenth passage in S.A. broth (S.A. dilution 1:3,000) had been reduced from a virulence of 0.00001 to a recorded virulence of 0.1 ml as measured by a single test. This culture was used for the following tests.

Seven passages were made at intervals of about 2 days in blood broth, and, at the end of the seventh passage, the virulence of the final culture was titrated in mice, duplicate tests being made for each culture dilution. The results were as follows:

MOUSE NO	AMOUNT OF INOCULUM*			
	0.2 ml	0.1 ml	0.05 ml	0.02 ml
a	D	D	L	L
b	D	D	L	L

* Sixteen-hour culture in blood broth.

It is shown that the virulence was not regained but remained at the original level (MLD, 0.1 ml). From these and similar tests it was concluded that there was no indication of "regeneration" of virulence of the P-13 culture under the conditions of this experiment. Further mouse passages were not employed. The loss of virulence determined by continued contact with the drug was permanent—at least within the method and time limitations of the test.

Relation of bacteriostasis to the attenuating factor. Detailed observations on the bacteriostatic influence of sulfanilamide in the dilutions employed in this work on the virulent Conzello smooth culture was not an intentional part of the present study, and plate counts were not employed for recognizing bacteriostatic action. In the preliminary tests made for the purpose of gauging the initial drug dosage some bacteriostasis was observed, and its visible effect was naturally increased as the number of organisms in the inoculums was decreased. It may be said, however, that in the actual tests, while the culture was being passed gradually from an S.A. concentration of 1:10,000 to one of 1:100, there was no interruption of growth due to bacteriostatic action, nor was difficulty ever encountered in establishing growth in the tube of next stronger sulfanilamide concentration in the serial passages. Even in the passages in S.A. 1:100 dilution the growth developed as quickly and eventually became as voluminous as in any earlier tubes in the series involving a lesser concentration of the drug.

Thus the impression was gained that, under the conditions of our experiments, the bacteriostatic influence of the drug on the primary, virulent, smooth culture was slight. It was considerably less, in fact, than was shown by similar concentrations on a mucoid strain of a beta hemolytic streptococcus studied earlier by one of us (P. H.) with Faith P. Hadley (1941). On a later occasion we anticipate dealing with this aspect of the problem of intra-phasic and inter-phasic variation in virulence with special reference to the mucoid phase of a greening streptococcus of high mouse virulence. The examination of a considerable amount of clinical material in this hospital over the past ten years has left us with the impression that the common belief in the low virulence of the alpha and gamma streptococci in general would undergo some modification if, at times of isolation from the patient, more attention were given to the identification of organisms in the mucoid phases—which, unfortunately, are rather rare in the general run of clinical material.

DISCUSSION AND CONCLUSIONS

The foregoing experiments and observations had a rather narrow aim—namely, to ascertain whether attenuation of the high virulence of the Conzello smooth culture, if it resulted when the organisms were placed in fairly continuous contact with sulfanilamide in broth mediums, occurred within the limitations of intra-phasic variation, as had the earlier enhancement of virulence of the same culture as the result of a long series of passages through mice; or whether, on the contrary, the loss of virulence would be associated with a transformation to the rough phase, as frequently happens under various experimental conditions in bacterial species of the *Bacteriaceae* and *Bacillaceae*.

The problem as a whole, however, when joined with other studies of a somewhat similar nature conducted by ourselves or others, also has a bearing on several other subjects: (1) the relative importance of intra-phasic and of inter-phasic variations in virulence in the determination of observed grades of virulence, the potentialities and limitations of virulence in a given species; (2) the manner of influence of sulfonamide drugs in effecting either one or the other of these two kinds of cultural modification, and (3) the possible significance of the commonly observed low grade of virulence encountered in cultures of greening and indifferent streptococci isolated from the blood stream or valvular lesions of patients with subacute bacterial endocarditis. These matters will now be briefly considered.

From the results presented in the foregoing pages it is possible to observe that the influence of the sulfanilamide in fairly high dilutions in broth mediums on the virulent, smooth-phase Conzello culture *in vitro* was such as to determine a rapid attenuation of virulence without, at the same time, bringing about notable growth-limiting effects. A considerable degree of attenuation was present in the culture from the fourth passage in 1:10,000 sulfanilamide dilution and was still more marked after two additional passages in dilution 1:5,000. This degree of attenuation, as well as the greater degree observed later, occurred without any accompanying consistent or long-maintained change in colony form, cell morphology, or cultural growth—with the exception of the manner of growth in broth mediums, as referred to on a previous page. There was no significant change in culture phase.

Thus, the attenuation of the culture in the present case, just as the exaltation of virulence of the same strain by mouse passage at an earlier date, as described by us in the first paper of this series (1943), was associated with what we then termed *intra-phasic variation*. The present results indicate that it is not necessary for sulfanilamide (or presumably other sulfa drugs) in contact with a culture *in vitro* to exert either marked cultural modifications or appreciable bacteriostasis in order that the virulence or invasiveness of the organisms may be greatly reduced. This attenuation again involves intra-phasic variation. These results were obtained with a greening streptococcus in smooth phase, an organism whose dissociative study has proved very difficult when compared, for example, with the hemolytic streptococcus and pneumococcus. To what extent similar results might appear in respect to other bacterial species or other culture phases of the same species is at present problematical. Some observations on this subject have, however, been made.

It has been reported by several investigators that gradual adaptation of organisms to a sulfonamide drug both *in vivo* and *in vitro* may be accomplished under conditions in which the organisms retain their original mucoid state, their original cell morphology, and all or much of their original virulence. Particularly has this appeared to be true in cases of lobar pneumonia and in pneumococcal meningitis.

McLeod and Daddi (1939) reported producing a sulfapyridine-fast strain of the pneumococcus in mucoid phase as a result of serial passages of the culture through increasing concentrations of the drug in serum broth. The organisms eventually derived were modified in certain metabolic and biochemical attributes but were not changed with respect to culture phase (colony form), cell morphology, or virulence, which remained at an MLD of 0.00001 ml for mice. The experimental procedure in this case involved 33 passages in sulfapyridine serum broth having a drug concentration of 1:160,000 to 1:16,000. At the end of this series 30 additional passages in broth not containing the drug failed to modify the acquired drug-fast attribute. The same was true after 10 transfers in normal mice. The culture modification seemed to be "permanent."

McKinney and Mellon (1941) studied, particularly *in vivo* but also to some extent *in vitro*, the effect of sulfonamide compounds on pneumococci from mice experiencing experimental pneumococcal peritonitis produced "by less than maximally efficient doses." The effect of the drug was to produce a graded series of "growth phases" beginning with a slightly modified mucoid colony and ending with a minute colony composed of unencapsulated, avirulent organisms possessing a variable cell morphology. Dwarf colonies and modified mucoid colonies were also observed. All of these variants showed modification in certain biochemical characters as well as in virulence. They were termed "modulations" and were regarded as representing a distinct category of variation differing from the M, S, and R dissociational pattern. The variants studied by McKinney and Mellon were not stable but, upon further passage, regained their capsules and characteristic pneumococcal morphology. Whether the original virulence was also regained was not tested. To the above it should be added that McKinney and Mellon, in an attempt to repeat the study of McLeod, passed a type 2 pneumococcus in the mucoid phase 27 times through serum broth containing concentrations of sulfapyridine from 1:100,000 to 1:1,000. They were able to confirm his results *in vitro* to the extent that their passage culture was not changed in cell morphology, type specificity, or virulence.

It appears likely that the lack of conformity between the results of McLeod and Daddi *in vitro* and those of McKinney and Mellon *in vivo* were due to differences in the environment of the organisms. Our present interest in the work of the investigators mentioned above lies, however, in the circumstance that neither pair was able to observe any changes of a definite dissociative character in the organisms submitted to the continued influence of the drug concerned.

The results were different in respect to the mucoid phase of a culture of beta hemolytic streptococcus studied earlier by one of us with Faith P. Hadley (1941). It was then pointed out that continued contact of this organism with sulfanilamide *in vitro* caused a phasic transformation from mucoid to smooth; and that, associated with this transformation, the primary virulence of the mucoid form was largely lost. This change furnished an example of what we have termed interphasic variation in virulence because the loss of virulence involved a transformation in culture phase. The results agreed with others observed when a culture transforms from mucoid to smooth or, perhaps, in other pathogenic species, from smooth to rough.[2] To what extent, in the experiments just referred to dealing with the hemolytic streptococcus, there may have been some reduction in virulence while the organisms were still in the mucoid phase was unfortunately not tested.

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[2] In present references to the culture phases of the pneumococcus we employ the terminology of Dawson rather than the inaccurate terminology of earlier work on dissociative variation in this species.

In surveying the results of McLeod and of McKinney and Mellon, together with the results of the earlier study on the hemolytic streptococcus and of the present study on a greening streptococcus, it is apparent that there are almost no common grounds capable of supporting any sort of general conclusion relating to the nature of the influence of sulfonamides on growing bacterial cultures so far as the part played by dissociative variation is concerned. One might readily have anticipated on theoretical grounds, and to some extent on the basis of earlier experimental observations, that the continued influence of a sulfa drug on virulent microorganisms either *in vitro* or *in vivo* would result in forcing a transformation in culture phase; and that such a transformation, whether it concerned the M to S transition or the S to R transition, would afford the most reasonable superficial explanation for the loss of virulence and the increase in phagocytability. In contrast to this result, it is apparent that among the studies referred to above in only one instance—namely, that of the beta hemolytic streptococcus—was there observed a clear-cut dissociative transformation accompanied by loss of virulence. This transformation was from virulent mucoid to slightly virulent smooth. The "modulations" observed in the pneumococcus by McKinney and Mellon had some resemblances to modifications that might be interpreted on the basis of dissociation in the accepted sense, but a definite transformation from mucoid to smooth phase was never observed. And the mucoid pneumococcus of McLeod, as also our smooth form of greening streptococcus, possessed an unexpected degree of cultural stability.

It is possible that the lack of conformity in the results of the various studies referred to above was to be anticipated—particularly when one considers the differences in respect to the bacterial species, the culture phase, the cultural environment, and the methods for effecting contact between organisms and drug. We are not able at present to decide what point of difference in the cultures or the technique is likely to be of greater significance, but we have made one observation, very likely made also by others, that may have a bearing on this aspect of the problem. In the performance of experiments not here reported it has sometimes seemed that the speed of increase in concentration of the drug with which the organisms are placed in contact is an influential factor. If first contact is with a drug of fairly strong concentration and the increase in concentration is rapid, a phasic transformation is more likely to result. If, on the contrary, the initial dose is weak and the increases are made slowly, it seems that the organisms will react by adaptation rather than by dissociative transformation. It is possible that this difference is one cause of the discrepancy between McLeod's results with the pneumococcus and the results with a mucoid beta hemolytic streptococcus reported earlier by one of us (1941). It is also clear, however, that our present results with the virulent Conzello smooth do not fit into either of those pictures. In the Conzello case the build-up in concentration of sulfanilamide could presumably have been more rapid and the smooth-to-rough transition perhaps observed. It is hoped to make a test of this possibility; also to study the influence of contact with this drug on a highly virulent, greening streptococcus in the mucoid phase. As stated earlier, we were not successful in producing this phase of the Conzello strain.

Although the present as well as the earlier paper in this series, both dealing with one aspect of the conditions underlying streptococcal virulence, do not justify extensive generalizations, taken together and along with an earlier study by one of us with Faith P. Hadley, they have a bearing on the subject of the influence of dissociative variation and, *pari passu*, the culture phases, in determining the range of expression of this attribute.

In an earlier publication one of us (Hadley, 1939) expressed the belief that "dissociative variation exerts a stronger determining influence on virulence than does any other single factor." And this was regarded as true because the dissociative culture phase present in a culture at a given time establishes and limits the range of actual virulence.[3] This is illustrated by the now well recognized fact that, among many pathogenic bacterial species, especially those belonging to the *Coccaceae*, *Bacteriaceae*, and, in some measure, the *Bacillaceae*, the most virulent form is the mucoid, whereas the rough is the least virulent. The smooth-phase culture occupies, with respect to virulence, an intermediate place. Among the pneumococci, the beta hemolytic streptococci, and the majority of the greening and indifferent streptococci the smooth form possesses relatively slight virulence, but among species of the *Bacteriaceae* and *Bacillaceae* it possesses relatively more. The rough form begins to acquire pathogenic significance only when one reaches the *Bacillaceae*, and this significance becomes greater in the *Mycobacteriaceae* and the *Actinomycetaceae*. All this means, in a general way, that transformations from rough to smooth to mucoid are most likely to be accompanied by increase in virulence, whereas transformations from mucoid to smooth to rough are most likely to be accompanied by decrease in virulence. These changes in culture phase in either direction, determining changes in virulence, involve what has been termed inter-phasic variations in virulence. When consideration is given to all three of the chief culture phases, M, S, and R, the range of virulence may be considerable, whereas within the limitations of a single culture phase the range has usually been regarded as slight.

Although the study of a considerable number of pathogenic bacterial species has thus enabled us to gain some understanding of the relationship between virulence and culture phase, what we have not learned so clearly, at least from experiments especially designed to throw light on this aspect of the subject, is the extent to which virulence can vary within a single, "pure" culture phase, despite the circumstance that the virulence of the same strain might also be varying in accordance with its transformations from one phase to another.

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[3] That all attempted evaluations of bacterial virulence give only relative values which may be modified by host resistance and other factors is, of course, taken for granted in this discussion. We are dealing here only with certain intrinsic factors lying in the organisms themselves.

With reference to this question the results of the present study, as well as of the study presented earlier, were somewhat unexpected. For it had seemed reasonable to anticipate that any marked increase in virulence of the Conzello smooth culture would be accompanied by transformation to the mucoid phase, as in the pneumococcus and beta hemolytic streptococcus, and, conversely, that any considerable decrease in virulence of this culture might well be accompanied by transformation to or toward the rough phase.

As a matter of fact, neither of these things happened; for the culture throughout the entire earlier period of exaltation of virulence by mouse passage (first paper), as well as during its later period of attenuation by passage in sulfanilamide broth, remained steadfastly in the smooth phase. Although, earlier, we did produce the rough form by other methods and observed its low virulence (0.7 ml for mice), we were unable to produce the mucoid even at the moment of highest virulence. Thus all observed variations in virulence were strictly intra-phasic.

Whether this unusually broad range of intra-phasic variation in virulence is characteristic of greening streptococci in general or is limited to certain species remains for the present a question. One may tentatively conclude, however, that, although the predominant culture phase present in a culture at a given time determines in a broad way the extent to which exaltation of virulence is possible, in some streptococcal species existing in the smooth phase a considerable range of variation in virulence is possible within that phase.

In connection with the present study, it may finally be pointed out that the mouse virulence of the organisms after long contact with sulfanilamide in 1:100 dilution was about 100 times less than that of the culture at the time of isolation from the patient, and about 200,000 times less than when the culture possessed maximum virulence directly following the series of mouse passages reported in the earlier paper. It is thus apparent that, even at the time of isolation, the virulence was considerably above that commonly observed in alpha or gamma streptococci from cases of endocarditis lenta. But it also becomes clear that this culture not only could be greatly enhanced in virulence by mouse passage but also could be reduced to a very low order of virulence such as is more often associated with cultures of greening or indifferent streptococci from this disease. It is not known to what extent other similar streptococci from the same source possess so high a grade of potential virulence. Or why, if they do possess it, the fact is not more commonly revealed in the clinical course of the disease.

In this connection it is perhaps worth while taking into account the possibility that cultures of greening or indifferent streptococci isolated from the blood stream or heart valves in cases of endocarditis lenta are not so likely to be found in a strictly "normal" state as are similar cultures isolated from other infections that ordinarily have a briefer duration and are not accompanied by such a degree of development of specific antibodies as is observable in endocarditis.[4] It has often been observed that pathogenic organisms cannot grow continuously for long periods in the presence of their homologous immune serum without being modified in some respect and in some degree by the antibody content, low though its titer may be. These modifications include primarily enforced loss of virulence, but they may also involve other attributes of the culture. Observations of this sort serve to increase the probability that, at the time when the first infection of the heart valves in endocarditis lenta occurs, the organisms concerned possess a degree of virulence considerably greater than that capable of demonstration in cultures isolated when the case first comes to the attention of the attending physician.

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[4] In blood samples from patients experiencing subacute bacterial endocarditis we have observed titers against one or another variety of greening or indifferent streptococci running from 1:160 to 1:1,280.

SUMMARY

The first paper in this series showed that the virulence of a culture of greening streptococcus in the smooth phase and of low virulence, isolated from a hospital patient dying of subacute bacterial endocarditis, could be greatly enhanced while the culture remained in the same phase. This was referred to as an instance of *intra-phasic* variation in virulence as contrasted with *inter-phasic* variation, the latter involving transformations in culture phase.

The present paper shows that this derived, virulent culture, when submitted to serial passages in increasingly strong concentrations of sulfanilamide in a broth culture medium, progressively lost its high virulence while, at the same time, retaining the same culture phase. The manner of growth gave no evidence of any marked bacteriostasis. Thus in both instances wide fluctuation in virulence occurred without necessity for the organisms to enter either the mucoid or the rough phase. The widest fluctuation was, however, associated with interphasic variation involving, in this case, the rough but not the mucoid.

The significance of these observations in relation to one mechanism of sulfanilamide therapy, together with their possible relation to the problem of virulence and to the infective process in endocarditis, is briefly considered.

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NUTRITIONAL REQUIREMENTS OF BACILLUS ALVEI AND BACILLUS PARA-ALVEI[1]

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Although bacteria associated with foul brood of bees have been studied by many investigators, very little was done with regard to the specific nutritional requirements of these organisms until it was demonstrated (Lochhead, 1942) that *Bacillus larvae*, etiological agent of American foul brood, required thiamine for growth. The studies have been extended at this laboratory to include investigations of the growth requirements of bacteria usually associated with foul brood disease but not necessarily the causal agents. The results of studies on the requirements of two strains of *Streptococcus apis* are presented in another paper (Katznelson, 1947). The present paper deals with *Bacillus alvei*, an organism repeatedly isolated from bee larvae affected with European foul brood but with no clearly established pathogenic properties, and *Bacillus para-alvei*, isolated from larvae affected with "para-foulbrood" disease of bees (Burnside, 1932; Burnside and Foster, 1935). Some question has arisen as to the distinction between this organism and *B. alvei*. Tarr (1936), on comparing their general morphological, cultural, and biochemical characteristics, concluded that the only distinguishing feature was a purely morphological one, involving change in the shape of the vegetative cells during sporulation and in the shape of the spores. Smith *et al.* (1946) considered the two organisms identical. It was hoped, therefore, that a study of the growth requirements of these bacteria might throw some light on their relationship in addition to furnishing information concerning their specific nutritional needs.

EXPERIMENTAL METHODS

Four stock strains of *B. alvei* and one of *B. para-alvei* were available for study at the outset; subsequent tests included six freshly isolated strains of *B. alvei* and two strains of *B. para-alvei*.[3]

To double strength basal medium containing 5.0 g glucose per liter and inorganic salts (Lochhead, 1942) were added various combinations of nitrogenous materials of varying complexity and vitamins of the B complex, and the solution was diluted to the desired volume. The medium was then adjusted to a pH of 6.8 to 7.0, dispensed in 8-ml amounts, and autoclaved for 15 minutes at 15 pounds' pressure. Inoculum was prepared by suspending cells from a 24-hour nutrient agar culture in 0.9 per cent saline, until a faint turbidity was obtained;

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[1] Contribution No. 225 (Journal Series) from the Division of Bacteriology and Dairy Research, Science Service, Department of Agriculture, Ottawa.

[2] Agricultural Scientist and Dominion Agricultural Bacteriologist, respectively.

[3] Kindly supplied by N. R. Smith, USDA, Beltsville, Md.

one 1-mm loopful was used per tube of medium. Growth was determined visually after 48 and 96 hours' incubation at 37 C. All glassware was scrupulously cleaned (acid-dichromate) and rinsed.

The following chemicals were tested:

Vitamins (μg per liter)

Thiamine	200	β-Alanine	200
Riboflavin	200	*para*-Aminobenzoic acid	200
Pyridoxine	200	Biotin	0.1
Pantothenic acid	200	Folic acid concentrate[4]	10
Nicotinic acid	200	Inositol	50 mg

Amino acids (mg per liter)

Glycine	80	*l*-Tryptophane	200
dl-Alanine	80	*l*-Cystine	250
dl-Valine	320	*dl*-Lysine·2HCl	400
l-Leucine	100	*l*-Histidine·HCl	160
l-Aspartic acid	100	*d*-Arginine·HCl	160
d-Glutamic acid	500	*l*-Proline	200
dl-Serine	80	*dl*-Isoleucine	200
dl-β-Phenylalanine	200	*dl*-Methionine	160
l-Tyrosine	140	*l*-Asparagine	500

EXPERIMENTAL RESULTS

Vitamin requirements. An attempt was first made to grow the organisms in media containing different sources of nitrogen with and without the vitamins of the B complex. The results (table 1) indicate that only casein hydrolyzate plus vitamins, or yeast extract, permitted growth of all strains; simpler nitrogenous compounds were ineffective. By omitting each of the 10 vitamins singly or in groups from a mixture of all in a casein hydrolyzate medium, it was found that thiamine was essential for growth. When amino acids were substituted for casein hydrolyzate, similar results were obtained with *B. alvei* strains (table 2). However, it was found repeatedly that *B. para-alvei* grew moderately well in the amino acid mixture alone, but was apparently stimulated by added thiamine (table 3).

Amino acid requirements. Each acid was omitted from a mixture of 18 in the basal medium containing thiamine. None of the acids appeared to be essential, although some were stimulatory at 96 hours. After further work on various combinations of acids the number required to give good growth of all strains was reduced to 14. When each one of these was omitted singly from the mixture, it was found that the omission of glycine, leucine, and cystine reduced growth of *B. alvei* strains appreciably (table 2). The omission of glycine from a medium containing thiamine markedly depressed growth of all three strains of *B. para-alvei* (table 3). In the absence of this vitamin, growth was also reduced when valine, phenylalanine, and isoleucine were left out of the medium, and was almost negligible in tubes devoid of cystine.

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[4] Kindly supplied by R. J. Williams, University of Texas, as a concentrate, "potency 3,100."

Subsequently, 6 fresh strains of *B. alvei* were isolated and tested for thiamine and amino acid requirements. At the same time an attempt was made to substitute $(NH_4)_2SO_4$ for some of the amino acids in a medium containing glycine, leucine, and cystine. The treatments and results obtained are given in table 4.

TABLE 1

Growth of Bacillus alvei and Bacillus para-alvei in media containing different sources of nitrogen and B vitamins

ADDENDA TO BASAL MEDIUM	GRAMS PER LITER	B. ALVEI STRAINS				B. PARA-ALVEI
		127	179	343	408	316
$(NH_4)_2SO_4$	3.0	−	−	−	−	−
$(NH_4)_2SO_4$ + vitamins		−	−	−	−	−
KNO_3	2.0	−	−	−	−	−
KNO_3 + vitamins		−	−	−	−	−
Asparagine	1.0	−	−	−	−	−
Asparagine + vitamins		−	−	−	−	−
Casein hydrolyzate	4.0	−	−	−	−	−
Casein hydrolyzate + vitamins		+++	++++	+++	+++	+++
Yeast extract	4.0	++++	++++	++++	++	++++

++++ = maximum turbidity at 96 hours.
− = no growth.

TABLE 2

Thiamine and amino acid requirements of B. alvei

ADDENDA TO BASAL MEDIUM	127	179	343	408
(1) Casein hydrolyzate	−	−	−	−
(2) Casein hydrolyzate + thiamine	+++	+++	+++	+++
(3) 18 amino acids	−	−	−	−
(4) 18 amino acids + thiamine	+++	+++	+++	+++
(5) 14 amino acids + thiamine	+++	+++	++++	+++
(6) (5) − Glycine	+	+	+	++
(7) (5) − Alanine	++	+++	+++	+++
(8) (5) − Valine	++	++	+++	+++
(9) (5) − Leucine	+	+	+	+
(10) (5) − Aspartic acid	++	++	+++	+++
(11) (5) − Glutamic acid	+++	++	+++	++
(12) (5) − Serine	+++	++	+++	+++
(13) (5) − Phenylalanine	++	++	++++	+++
(14) (5) − Tyrosine	+++	++	+++	++++
(15) (5) − Tryptophane	++	++	+++	+++
(16) (5) − Cystine	+	++	+	−
(17) (5) − Histidine	+++	++	++++	+++
(18) (5) − Arginine	++	++	++++	+++
(19) (5) − Methionine	++	++	++++	++++

TABLE 3

Thiamine and amino acid requirements of B. para-alvei

ADDENDA TO BASAL MEDIUM	STRAINS					
	316		551		552	
	+B₁	−B₁*	+B₁	−B₁	+B₁	−B₁
(1) Casein hydrolyzate	+++	−	+++	−	+++	−
(2) 18 Amino acids	+++	++	+++	++	+++	++
(3) (1) − Glycine	+	±	+	±	+	±
(4) (2) − Alanine	+++	++		++		++
(5) (2) − Valine	+++	+	+++	+	+++	+
(6) (2) − Leucine	+++	+	+++	++	+++	++
(7) (2) − Aspartic acid	+++	++		++		++
(8) (2) − Glutamic acid	+++	++		++		++
(9) (2) − Serine	+++					
(10) (2) − Phenylalanine	+++	±	+++	+	+++	+
(11) (2) − Tyrosine	+++	++		++		++
(12) (2) − Tryptophane	+++	++		++		++
(13) (2) − Cystine	+++	−	+++	±	+++	±
(14) (2) − Lysine	+++	++		++		++
(15) (2) − Histidine	+++	++		++		++
(16) (2) − Arginine	+++	++		++		++
(17) (2) − Proline	+++					
(18) (2) − Isoleucine	+++	+	+++	+	+++	+
(19) (2) − Methionine	+++					
(20) (2) − Asparagine	+++	++		++		++

± = very faint growth.

* Methionine, serine, and proline not included in −B₁ medium.

TABLE 4

Growth of various strains of B. alvei and of B. para-alvei in media of different composition

ADDENDA TO BASAL MEDIUM	B. ALVEI STRAINS										B-PARA ALVEI
	127	179	343	408	NS1	NS2	NS3	NS4	NS5	NS6	316
(1) 18 Amino acids	−	−	−	−	−	−	−	−	−	−	++
(2) 18 Amino acids + thiamine	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++
THIAMINE ADDED TO BASAL MEDIUM											
(3) Glycine + cystine + leucine*	+	+	++	+	+	+	++	−	+	++	++
(4) (3) + $(NH_4)_2SO_4$	+++	++	++	++	++	++	+++	+++	+	++	++
(5) 14 Amino acids†	+++	++	++++	++++	++++	+++	++++	+++	++	++++	+++
(6) Acids in (1) − glycine	−	+	+	++	+	+	−	−	+	++	++
(7) Acids in (1) − cystine	−	++++	−	−	++++	−	++++	++	−	−	+++
(8) Acids in (1) − leucine	+++	+	++++	++++	++	+++	+++	++++	+++	++++	+++
(9) Yeast extract	+++	+++	++++	++++	++	+++	++++	++	++	++++	+++

* Double the concentration given in text.

† See table 2 for the list of these acids.

The original *B. alvei* and *B. para-alvei* strains were also included in this experiment. All the strains of *B. alvei* required thiamine and could grow in a synthetic medium containing this vitamin and 18 amino acids. Again *B. para-alvei* grew in the absence of added thiamine but was stimulated by it. Strains of *B. alvei* developed poorly in a medium containing glycine, cystine, and leucine, but were apparently stimulated by the addition of $(NH_4)_2SO_4$ to these acids, indicating that inorganic nitrogen can replace certain amino acids if the more important ones are available. However, 14 amino acids permitted growth of most of the strains, which growth was distinctly superior to that produced in the $(NH_4)_2SO_4$ medium and practically equivalent to that obtained with 18 acids. Again, glycine was stimulatory for, or required by, all the strains of *B. alvei*; cystine for most; and leucine for several. In table 2 it was shown that leucine was stimulatory for strains 127, 179, 343, and 408, whereas in the last experiment it was found to stimulate only one of these (179). It should be pointed out, however, that in the first experiment leucine was omitted from a mixture of 14 acids, whereas in the experiment reported in table 4 it was omitted from a mixture of 18. It is quite possible that among the four omitted was one which could substitute for leucine in the medium containing 18 acids, thereby obscuring the effect of omitting it which appeared when only 14 acids were used. The best growth of *B. para-alvei* was obtained with 14 or 18 amino acids in the presence of thiamine, the organism developing moderately well in a medium containing the vitamin and glycine, cystine, and leucine. It was not stimulated appreciably by $(NH_4)_2SO_4$, nor was it affected by omission of cystine or leucine; however, when glycine was left out, growth was depressed.

DISCUSSION

As the following tabulation indicates, the most striking difference between the nutritional requirements of *B. alvei* and *B. para-alvei* strains is the essentiality of thiamine for the former as compared to its purely stimulatory effect on the latter in the amino acid medium.

ADDENDA TO BASAL MEDIUM	GROWTH OF	
	B. alvei	*B. para-alvei*
Casein hydrolyzate	0	0
Casein hydrolyzate + thiamine	+	+
Amino acids	0	+
Amino acids + thiamine	+	+

The growth of *B. para-alvei* in the amino acid medium devoid of thiamine may be due to contamination with this vitamin of one or more of the amino acids used, but in that case it might perhaps be expected that *B. alvei* would also grow in this medium, which is not the case. It is conceivable, too, that *B. para-alvei* is more sensitive to smaller amounts of thiamine such as might be found in contaminating traces than is *B. alvei*. The possibility that the former can synthesize the vitamin from the amino acids used, whereas *B. alvei* cannot, also suggests itself. This problem is being studied further. If it can be proved that *B. para-*

alvei can actually satisfy its thiamine requirements by synthesis instead of depending on an external supply as does *B. alvei*, then a fundamental physiological difference between these two organisms will have been brought to light, which, when combined with the morphological differences noted by Tarr (1936), may strengthen the case for separating these bacteria into two species.

ACKNOWLEDGMENT

The authors are indebted to Miss C. M. Clark for technical assistance.

SUMMARY

Bacillus alvei and *Bacillus para-alvei* required thiamine when grown in a casein hydrolyzate medium. In a synthetic medium containing amino acids, all strains of *B. alvei* tested required thiamine for growth, whereas strains of *B. para-alvei* produced moderate growth in the absence of this factor.

B. alvei grew in a simplified medium containing thiamine and 14 amino acids, of which glycine, leucine, and cystine were essential or stimulatory, depending on the strain; whereas *B. para-alvei* was stimulated by glycine. However, when thiamine was omitted from this amino acid medium, certain other acids such as valine, phenylalanine, isoleucine, and particularly cystine also became stimulatory or essential for the growth of the latter organism.

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THE CULTIVATION OF BACTERIUM TULARENSE IN EMBRYONATED EGGS

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Since Goodpasture and Anderson's work in 1937, many investigators have cultivated a variety of bacterial species in embryonated eggs. The method has proved useful in the study of the pathogenesis of infectious diseases and offers a means for cultivation of organisms in the presence of living tissue The use of the embryonated egg as a source of material for vaccines is well established for the rickettsiae and viruses.

In tularemia a very solid immunity is established in man by infection, but prophylactic vaccination with killed cultures, while helpful, does not afford so solid an immunity as an attack of the disease. It might be surmised that an effective antigen is produced in the presence of living tissue that is not produced in artificial culture media. We were, consequently, interested in exploring the possibility that *Bacterium tularense* when grown in the presence of living cells in embryonated eggs might be a better antigen than when grown *in vitro*. Before undertaking this problem, however, it was deemed advisable to obtain certain data on the behavior and characteristics of the organisms in this milieu, in which it propagates readily (Buddingh and Womack, 1941; Ransmeier, 1943; Larson, 1945). The work reported here was done with the object of determining: (1) the degree of multiplication of *Bacterium tularense* in embryonated eggs; (2) the differences exhibited by strains of varying virulence when grown in eggs; (3) the effect of serial passage on virulence for mice and eggs; and (4) the survival of *Bacterium tularense* when stored in harvested egg material.

EXPERIMENTAL

Determination of the accuracy of plate counts. Since it was important to determine the number of organisms in the various tissues of the inoculated embryonated egg, a preliminary study was made to determine whether a reliable plate counting method could be devised. No such method is described in the literature, and the usual means of determining numbers of viable organisms is through titrations in mice. Since this latter method is both laborious and expensive, it was highly desirable to substitute a cultural method if possible.

Various modifications and substitutes were made in Francis' (1922) cystine blood medium. Substitution of cysteine for cystine was found to increase growth. The final pH of this medium was found to be important and the optimum pH to lie within a narrow zone around 6.9. Since phosphate ions inhibited growth, sodium hydroxide was used for adjustment of the pH. Of various peptones used, Difco peptone gave the best growth. Liver concentrate could not replace blood in glucose cysteine agar and interfered with growth when added to the whole medium containing blood. The following method of making glucose cysteine blood agar (hereafter called GCBA) proved to be very satisfactory.

Solution A. Double strength meat extract broth
0.6 per cent meat extract
2.0 per cent Difco peptone
1.0 per cent NaCl

Solution B. 3 per cent solution of agar in water

Solutions A and B were autoclaved separately and may be made in liter lots and stored. For use, 0.1 per cent cysteine-HCl is added to the broth (solution A) which must be at a temperature of not more than 30 C. The reaction is adjusted to pH 7.1 with NaOH and checked in a reliable potentiometer. An equal volume of melted double strength agar (solution B) is then added while hot, and thoroughly mixed. The cysteine agar is placed in convenient containers, sterilized at 15 pounds' pressure for 20 minutes, and cooled to 45 C. Five ml of sterile 50 per cent glucose solution and 3 to 5 ml of sterile defibrinated rabbit or human blood are added to each 100 ml of medium. The medium is poured into petri dishes and allowed to solidify. The finished plates should be uniformly bright red and free from surface moisture.

As plain cysteine broth or agar cannot be stored for more than 24 hours without loss of growth-promoting properties, the cysteine-HCl should be added just before making the final medium as described above. The finished plates, however, may be stored in the refrigerator and used for 3 weeks after preparation.

The method of inoculation is to pipette into the center of the plate 0.1 ml of the fluid to be counted. This fluid is then spread gently over the surface with a sterile, bent glass rod. After drying, the plates are incubated at 37 C for 48 to 72 hours and the colonies counted.

Nine virulent strains of *Bacterium tularense* have been tested in this medium and have given satisfactory plate counts. Eight strains of reduced virulence grew more slowly, and the plate counts were lower, irregular, and not reproducible. Statistical analysis of the accuracy of plate counts on this medium using the virulent Schu strain follows.[1]

Comparison was made of the GCBA plate count and the mouse LD_{50} titration as methods of estimating the number of *Bacterium tularense* in a given bacterial suspension. Three to four plates were inoculated with appropriate serial tenfold dilutions of the original suspensions, and 6 to 12 mice were injected intraperitoneally with 0.5 ml of each of 4 or 5 serial tenfold dilutions. The plate counts were expressed as the number of organisms per ml of the original suspension. The LD_{50} was calculated by the method of Reed and Muench (1938) and expressed as the number of LD_{50} per ml of the original suspension. By dividing the number of organisms per ml as determined by plate count by the number of

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[1] Since this work was done, Snyder *et al.* (1946) have described a medium used for the enumeration of *Bacterium tularense* which has been shown to be as reliable as the medium described in this paper but which has the disadvantage of being less stable than the glucose cysteine blood agar medium.

LD_{50} per ml as determined by mouse titration, a figure representing the number of organisms per LD_{50} was obtained. Over a period of two years 20 such titrations were made on bacterial suspensions in saline and 20 on bacterial suspensions in infected embryonated eggs. The mean number of organisms per LD_{50} in the 20 saline suspensions was 0.867, with a standard deviation of 0.7115 and a standard error of 0.1591. For the 20 embryonated egg suspensions the mean was 1.08, with a standard deviation of 0.8869 and a standard error of 0.1983. These figures indicate that the GCBA plate count and the mouse LD_{50} measure approximately the same end point. It is improbable that the figures would agree so closely if the end point were other than unity, and therefore it has been assumed that one organism gives rise to a colony on GCBA and also constitutes a lethal dose for a mouse. Clumps of organisms probably occur in the egg suspensions and might invalidate this conclusion; however, clumps of organisms almost never occur in saline suspensions as shown by direct microscopic examination of wet mounts.

TABLE 1

Multiplication of Bacterium tularense in 12-day-old embryonated eggs following yolk sac or c. a. membrane inoculation

ROUTE OF INOCULATION	GROWTH OF BACTERIUM TULARENSE IN			
	Embryo	C.a. membrane	Yolk sac	Allantoic fluid
	millions of organisms/ml of tissue			
Membrane	160	740	260	340
Yolk sac	172	1,380	8,200	620

All tissues were harvested 72 hours after inoculation.

The degree of multiplication in embryonated eggs. Embryos 8 to 12 days old were inoculated on the membrane or into the yolk sac in the usual manner. The inoculum consisted of 0.2 ml of a standard bacterial suspension prepared by the emulsification of a 24-hour growth from GCBA and matched to 40 per cent transmission in a Coleman spectrophotometer. This standard suspension contained from 1 to 2 billion viable organisms per ml as shown by plate count. The eggs were sealed with collodion-iodine and incubated at 35 to 37 C. The various tissues and allantoic fluid were harvested under aseptic conditions, and the membranes and yolk sacs were shaken with sterile beads in a shaking machine. Serial dilutions were made and plate counts were done by the method described above.

Quantitative studies were made on the yield of bacteria from various tissues after membrane or yolk sac inoculation of virulent strain Schu. Within 24 hours large numbers of organisms were demonstrable in all parts of the egg. The embryos usually died in from 72 to 120 hours after inoculation. Table 1 gives an example of the quantitative yield when the eggs were harvested 72 hours after inoculation.

These figures, which were confirmed by many experiments, show that the greatest multiplication of *Bacterium tularense* took place in the yolk sac of yolk-sac-inoculated eggs. In all cases the embryo gave the smallest yield of organisms. It is apparent from these results that the number of organisms in either the membrane, allantoic fluid, or yolk sac was sufficient to serve as a source of vaccine material. Vaccines, accordingly, were prepared from various tissues, and the results of the use of these vaccines will be presented in a later communication.

Relative virulence of strains of Bacterium tularense for embryonated eggs. Eight fully virulent strains and 7 strains of diminished virulence were cultivated in 8-day embryonated eggs. An attempt was made to determine the relative virulence of the strains for the chick embryo and to correlate the size of the inoculum with the time of death and the viable count, and to determine the effect of continued storage at incubator temperature on the count after death of the embryo.

The strains to be used were grown for 24 hours on GCBA, and the growth was emulsified in physiological saline. The turbidity of the suspension was adjusted to give a count of 1 to 2 billion organisms per ml. From this suspension logarithmic dilutions were made in saline for the inoculation of the eggs. Twenty eggs were inoculated with each dilution. Eggs were candled daily, and the number of dead embryos was recorded. The first 10 eggs containing dead embryos were harvested on the day of maximum embryo death. The remaining 10 eggs were left in the incubator at 37 C and harvested 2 to 6 days later. Harvesting was done by mixing the entire contents of 10 eggs in a Waring "blendor" for 3 minutes. The foam was allowed to settle for 5 minutes, then logarithmic dilutions of the liquid portion were made in saline and counted on GCBA plates.

The results are shown in tables 2 and 3. The 8 virulent strains killed chick embryos through the 10^{-8} dilution of inoculum. The maximum embryo deaths occurred on the third to fourth day with the heavy inoculum and on the sixth to ninth days with the light inoculum. Of the 8 virulent strains studied, 6 showed greater multiplication in eggs receiving the heaviest inoculum, whereas 2 strains, Holt and Schu, showed approximately the same degree of multiplication in all dilutions of inoculum. In practically all cases storage of dead embryos in the incubator for 2 to 6 days after death resulted in a decrease in the number of viable *Bacterium tularense.*

The 7 less virulent strains killed chick embryos more slowly. Multiplication of *Bacterium tularense* was not so great as with the majority of virulent strains and was about the same for all dilutions of inoculum. At the level of the 10^{-7} inoculum an average of the yield of the virulent strains was a little more than 3 times that of the less virulent strains.

The data on strain 38 are not shown in table 3 because the results were so variable on repeated titrations. Although large inocula of strain 38 are not lethal for mice, a small percentage of chick embryos were killed by heavy inocula. Fewer than 50 per cent of 8-day embryos were killed by the inoculation of 0.2 ml of an undiluted standard suspension containing 400,000,000 organisms. In eggs inoculated with dilutions of 10^{-2} to 10^{-6} of strain 38 there were scattering deaths on the fifteenth to the eighteenth days.

TABLE 2

Titration of eight virulent strains of Bacterium tularense in 8-day embryonated chicken eggs

STRAIN	MOUSE LD$_{50}$*	DIL. OF INOCULUM	DEATH / TOTAL NO.	DAY OF MAX. EMBRYO DEATH	PLATE COUNT PER ML OF 10 WHOLE EGGS (× 10^8)		CHICK EGG LD$_{50}$*
					Day of max. embryo death	10 days after inoculation	
Carr	$10^{-9.5}$	10^{-2}	20/20	4	15.8	10.3	10^{-8+}
		10^{-4}	20/20	4	5.5	.45	
		10^{-6}	19/20	5	7.4	1.52	
		10^{-8}	19/20	6	2.4	3.8	
Dieck	$10^{-9.6}$	10^{-2}	20/20	4	34.5	.85	10^{-8+}
		10^{-4}	20/20	6	17.9	1.85	
		10^{-6}	20/20	6	10.8	2.0	
		10^{-8}	18/20	9	3.2	3.1	
Camp	$10^{-9.5}$	10^{-2}	20/20	4	41.2	.97	10^{-8+}
		10^{-4}	20/20	5	8.9	.4	
		10^{-6}	20/20	7	7.4	.8	
		10^{-8}	20/20	7	13.2	.8	
Ince	$10^{-9.5}$	10^{-2}	20/20	4	21.4	C	10^{-8+}
		10^{-4}	20/20	5	24.5	C	
		10^{-6}	18/20	6	21.5	3.94	
		10^{-8}	16/20	7	8.0	—	
Holt	$10^{-10\ 6}$	10^{-2}	20/20	6	12.7	7.1	10^{-8+}
		10^{-4}	20/20	6	14.3	9.9	
		10^{-6}	20/20	6	13.5	10.7	
		10^{-8}	18/20	6	15.2	11.9	
Scherm	$10^{-9.2}$	10^{-2}	20/20	3	7.5	.95	10^{-8+}
		10^{-4}	20/20	4	5.6	1.4	
		10^{-6}	20/20	5	1.1	2.6	
		10^{-8}	18/20	7	2.3	3.3	
Coll	$10^{-8.8}$	10^{-2}	20/20	4	7.1	1.4	10^{-8+}
		10^{-4}	20/20	4	6.0	1.0	
		10^{-6}	20/20	5	2.7	1.0	
		10^{-8}	17/20	6	—	4.6	
Schu	$10^{-9.5}$	10^{-2}	20/20	3	3.4	1.3	10^{-8+}
		10^{-4}	20/20	4	4.0	.9	
		10^{-6}	20/20	5	3.9	1.0	
		10^{-8}	18/20	6	—	4.0	

C = contaminated.

— = no count made.

* LD$_{50}$ expressed as the dilution of a standard suspension which killed 50% of the embryos or mice.

Plate counts made on pools of 10 inoculated eggs, 3 days and 10 days after inoculation, gave extremely irregular results. Cultures of strain 38 could be recovered from the eggs in most cases, but the counts were inconsistent and the number of colonies on the plates usually was less than the number of organisms inoculated. This would indicate that the organisms did not multiply, but survived fairly well.

These results on strains of varying virulence, however, indicate that inoculation into 8-day embryonated eggs does not furnish an accurate method for detecting

TABLE 3

Titration of six Bacterium tularense strains of low virulence in 8-day embryonated chicken eggs

STRAIN	MOUSE LD$_{50}$*	DIL. OF INOCULUM	DEATH / TOTAL NO.	DAY OF MAX. EMBRYO DEATH	PLATE COUNT PER ML OF 10 WHOLE EGGS (× 10^6)		CHICK EGG LD$_{50}$*
					Day of max. embryo death	10 days after inoculation	
Jap	$10^{-4.4}$	10^{-2}	20/20	5	19.4	C	10^{-8+}
		10^{-4}	20/20	6	19.2	10.2	
		10^{-6}	18/20	6	20.4	20.5	
		10^{-8}	17/20	9	—	24.0	
Pier	$10^{-1.0}$	10^{-2}	20/20	6	6.0	4.2	10^{-8+}
		10^{-4}	20/20	6	7.1	3.0	
		10^{-6}	20/20	6	5.4	5.4	
		10^{-8}	18/20	7	5.8	8.0	
Russ	$10^{-2.0}$	10^{-2}	16/20	7	3.4	1.5	10^{-8+}
		10^{-4}	17/20	8	4.0	4.0	
		10^{-6}	16/20	8	2.2	2.2	
		10^{-8}	16/20	8	4.6	—	
Max	$10^{-1.7}$	10^{-2}	21/21	4	3.6	C	$10^{-8.0}$
		10^{-4}	20/20	8	3.2	1.2	
		10^{-6}	20/20	9	10.6	8.0	
		10^{-8}	10/20	9	—	4.3	
Ohara	$10^{-1.0}$	10^{-2}	20/20	6	1.5	.025	$10^{-7.2}$
		10^{-4}	20/20	7	.6	.01	
		10^{-6}	19/20	8	1.6	.09	
		10^{-8}	4/20	10	—	.08	
26	$10^{-1.0}$	10^{-2}	20/20	5	.4	.03	$10^{-6.26}$
		10^{-4}	18/20	8	1.5	—	
		10^{-6}	11/20	8	.01	—	
		10^{-8}	3/20	0	—	—	

C = contaminated.

— = no count made.

* Expressed as dilution (of a T 40 *Bacterium tularense* suspension) which killed 50% of the embryos or mice.

differences in virulence between strains of *Bacterium tularense*. The differences in the chick LD_{50} are not so great as those observed in the mouse LD_{50}. Eight-day embryos were somewhat more susceptible than 12-day embryos to all strains of *Bacterium tularense*.

The growth of Bacterium tularense in nonviable embryonated eggs. Although Buddingh and Womack (1941) and we have shown that *Bacterium tularense* parasitizes the cells of the yolk sac, membrane, and embryo, it appeared of interest to determine how well, if at all, *Bacterium tularense* would grow in the embryonated egg if inoculation was made after the embryo was killed. If organisms grown in the embryonated egg were used as a source for vaccine preparation, it would be worth while to know whether eggs killed by the manipulation of inoculation would have to be discarded.

Forty-five eggs (7-day embryos) were inoculated with 0.2 ml of 10^{-2} dilution of a standard suspension of the Schu strain of *Bacterium tularense*. The eggs were divided into two lots of 20 and 25, respectively. Twenty were inoculated into the yolk sac as usual; 25 were killed at the time of inoculation by inserting the needle twice at an angle directed toward the embryo. The bacterial suspension was inoculated into the yolk sac at the third stroke. All eggs in this group containing viable embryos after 18 hours at 37 C were discarded.

All of the embryos infected in the usual manner were dead by the third day and were placed in the refrigerator. The group containing nonviable embryos was incubated at 37 C for 3 days, then placed in the refrigerator until harvested. After 1 day in the refrigerator the contents of both sets of eggs were harvested, and plate counts were made in triplicate. Three eggs were pooled for each count. In two experiments the average count in 7 pools of embryos allowed to die of infection was 2.74×10^9 per ml, whereas the average amount in 6 pools of embryos killed at the time of infection was 2.76×10^9 per ml. There were thus no significant differences between the multiplication of *Bacterium tularense* in living as compared with dead embryos.

Growth of Bacterium tularense in duck eggs. Since no reports are available on the growth of *Bacterium tularense* in eggs other than chicken eggs and since duck eggs might be expected to give a larger total yield because of their size, the following experiments were performed:

In the first experiment, sixteen 9-day-old embryonated duck eggs were inoculated with 0.2 ml of 10^{-2} standard suspension of the Schu strain. Twenty 11-day embryonated chicken eggs were inoculated in the same manner. Both lots of eggs were incubated at 37 C and harvested on the fourth day, after overnight storage in the refrigerator. Yolk sacs, membranes, and allantoic fluid were harvested separately. Yolk sacs from 14 duck eggs and 20 chicken eggs were pooled by species for a plate count. Membrane and allantoic fluid were also pooled before plating.

In this experiment the numbers of organisms per ml were as follows for the duck eggs: yolk sac, 22.60×10^9; membrane, 3.72×10^9; allantoic fluid, 2.14×10^9. In the chicken eggs the yolk sac count was 3.30×10^9; membrane, 2.58×10^9; and allantoic fluid, 0.35×10^9. In general, the counts obtained on infected duck eggs were higher than those on chicken eggs. In duck eggs, as in chicken eggs, the yolk sac contained a larger number of organisms than the membranes or fluids.

In a second experiment, 10 duck and 10 chicken eggs were inoculated into the yolk sac with serial dilutions of 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} made from a standard suspension. The average count for all dilutions of inoculum was 5.34×10^9 in the duck eggs and 3.99×10^9 in the chicken eggs. Again the average count was higher in the duck eggs, though the difference was not so great as in the first experiment. A third experiment gave yolk sac counts comparable to the second experiment, and we are unable to account for the extremely high numbers found in the duck egg yolk sacs in the first experiment. In these experiments the duck embryo survived an average of 1 day longer than the chick embryo.

Effect of serial egg passage on the virulence of Bacterium tularense. Since serial passage in eggs in the case of some viruses and the rickettsiae may alter their virulence for eggs and mice, it seemed worth while to study the effect of serial passage on the virulence of *Bacterium tularense* in embryonated eggs.

TABLE 4

The effect of serial egg passage on the virulence of Bacterium tularense

STRAIN*	LD_{50} TITER IN	
	Mice†	Chick embryo†
SM1	$10^{-9.22}$	$10^{-7.17}$
SYS26	$10^{-9.30}$	$10^{-9.32}$
SME10	$10^{-9.27}$	$10^{-9.0}$

* SM1 = mouse passage 1 (original culture; SYS26 = yolk sac passage number 26; SME10 = membrane passage 10.

† LD_{50} expressed as a dilution of a standard suspension that killed 50% of the embryos or mice.

A virulent strain of *Bacterium tularense*, Schu, was passed through the yolk sac and membrane of 12-day embryonated eggs, and the subsequent LD_{50} for mice and chick embryos was determined. Twelve-day eggs were chosen because of their greater resistance to infection. Table 4 shows the results of these experiments. No significant change in virulence for mice could be detected after yolk sac passage or membrane passage. This is in agreement with Larson (1945), who states that 13 yolk sac passages did not result in loss of virulence for mice. The virulence for the chick embryo seemed to be materially increased on yolk sac passage. The organism tended to die out on repeated membrane passage, but virulence titrations showed significant increase in virulence for chick embryos.

Survival of Bacterium tularense grown in embryonated eggs. The effect of prolonged storage of infected embryonated eggs upon the percentage of survival and the virulence of *Bacterium tularense* was determined as follows:

Sixty embryonated eggs were inoculated into the yolk sac with the virulent Schu strain. All eggs in which the embryo died on the third day were divided into three lots and stored in the shell at −40 C, at −4 to −6 C, and at room temperature. Two whole eggs were harvested before storage and shaken in a bottle

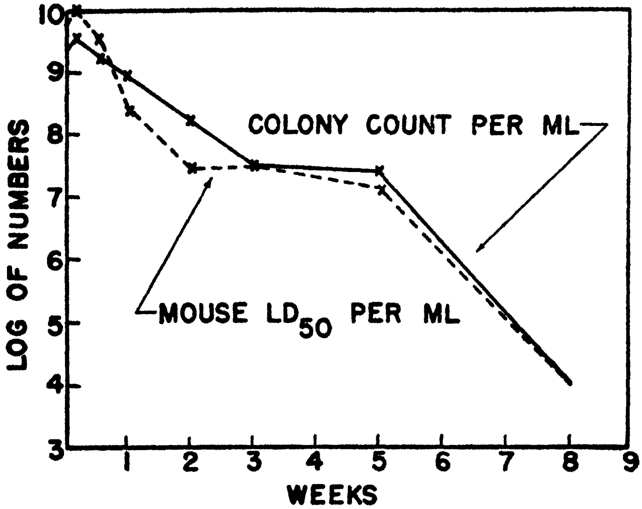

FIG. 1. SURVIVAL OF BACTERIUM TULARENSE IN INFECTED EMBRYONATED CHICKEN EGGS STORED AT ROOM TEMPERATURE

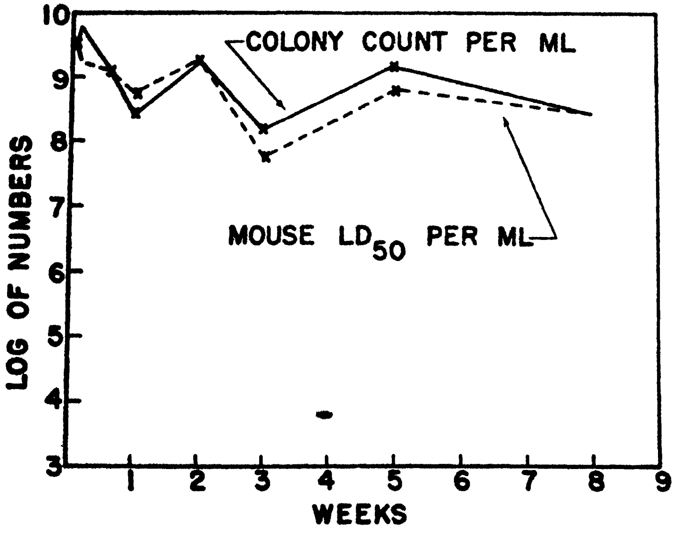

FIG. 2. SURVIVAL OF BACTERIUM TULARENSE IN INFECTED EMBRYONATED CHICKEN EGGS STORED AT −4 TO −6 C

with glass beads for 30 minutes. Plate counts and mouse titrations were done on these eggs and served as controls on the stored eggs. At intervals, 2 eggs were harvested from each stored lot and counts were made as above.

Figures 1, 2, and 3 give the survival time expressed as colony counts on GCBA plates and as mouse LD_{50} per ml. These results show that when tested after 35 days and after 56 days, the numbers surviving at −40 C and at −4 to −6 C were approximately equal. There was a rapid and progressive decrease in the numbers of organisms in eggs stored at room temperature. The decreasing numbers of organisms on storage as shown by plate count paralleled the number of mouse LD_{50}, indicating that the virulence of the surviving organisms was maintained. Further experiments showed that the death rate of *Bacterium tularense* in frozen eggs was not accelerated if the eggs were thawed, minced, and storage continued for 2 months in a liquid state at +4 to +6 C.

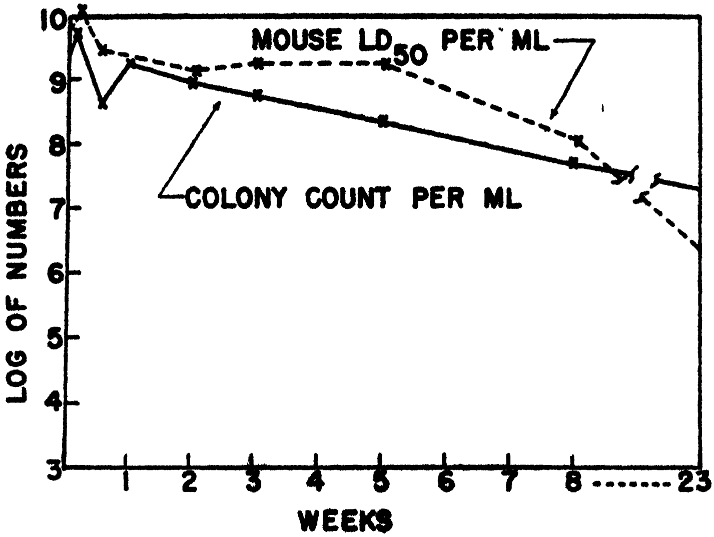

FIG. 3. SURVIVAL OF BACTERIUM TULARENSE IN INFECTED EMBRYONATED CHICKEN EGGS STORED AT −40 C

COMMENT

A statistical analysis of the plate counting technique described in this paper shows that it is as accurate as mouse titration. This method constitutes a useful tool that has enabled us to determine the number of organisms in embryonated eggs under varying conditions of experiment.

The results obtained by quantitative examination of embryonated eggs after infection with virulent *Bacterium tularense* indicate that this organism grows well in all parts of the embryonated egg. Our results, using more exact quantitative methods, are in agreement with those of Larson (1945), who used microscopic examination of the yolk sac material and titration in mice as a means of estimating the number of organisms in infected eggs. Buddingh and Womack (1941) and Ransmeier (1943), using membrane inoculation alone, observed microscopic evidence of abundant growth in the embryonated egg. Jawetz and Meyer (1944), who studied the infection of the chick embryo with *Pasteurella pestis*, found that when 20 to 2,000 avirulent *P. pestis* were injected, the average survival time of the embryo was greater than for those injected with virulent organisms, and a few of the chicks hatched. Our results showed a smaller difference between virulent and less virulent strains of *Bacterium tularense* since the average survival time of chick embryos infected with 8 virulent strains was 4.7 days, whereas the average survival time with 6 less virulent strains was 7.0 days. The average number of chicks surviving inoculation with all dilutions of the virulent organisms was 3 per cent, but the survival of chicks receiving the same number of less virulent organisms was 17 per cent. Jawetz and Meyer observed that there was no difference in survival if the eggs were inoculated with 200 million virulent or avirulent *P. pestis.*

The less virulent strains of *Bacterium tularense* did not multiply so abundantly as the virulent organisms. This is comparable to the results of Ransmeier, using 12-day embryos. The inability to multiply abundantly is also reflected in the growth of virulent and avirulent strains on plates. The completely avirulent organism 38 fails to give accurate plate counts and kills very few of the embryos. These results on the difference between virulent and less virulent *Bacterium tularense* are apparently due to the inability of the avirulent organisms to multiply, since the strains cannot be differentiated on the basis of colony formation, morphological characteristics, serological tests, or antigenic components.

Serial passage of virulent *Bacterium tularense* through eggs increased the virulence of the organism for chick embryos but not for mice. It is not known whether this represents any other adaptive change on the part of the organism. Storage in the frozen state or in the cold did not modify virulence of the surviving organisms, but the percentage of organisms surviving after 3 months was relatively small.

ACKNOWLEDGMENT

The authors wish to acknowledge the skillful technical assistance of Ruth T. Doiron.

SUMMARY AND CONCLUSIONS

An accurate surface plate count method for *Bacterium tularense* is described.

Bacterium tularense multiplies abundantly in the tissues and fluid of the embryonated chicken or duck egg.

Multiplication is greatest in the yolk sac, particularly after yolk sac inoculation.

The property of virulence affects the multiplication of the organism in the embryonated egg and the survival of the embryo.

The virulence of *Bacterium tularense* for chick embryos is enhanced on serial passage through embryonated eggs.

Storage in the frozen state or in the cold preserves, for a period of 3 months, approximately 1.0 per cent of the original number of organisms present.

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ACTINOMYCETIN

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It has been stated (Hoogerheide, 1944) that actinomycetin is not an antibiotic, on the ground that it is supposed to be inactive toward living bacteria. This appears to be a misinterpretation of our data or an overlooking of some of the facts established. It therefore calls for some explanation.

Since 1924 it has been well known that certain strains or species of actinomycetes are able to dissolve various bacteria, such as *Staphylococcus aureus*, in the living state (Gratia and Dath, 1924), as well as a wide variety of dead microorganisms. The former is no doubt an antibiotic effect.

Culture filtrates from broth cultures of these actinomycetes have a strong lytic action on suspensions of dead bacteria, but, in addition, they are able to cause a rapid lysis of a few bacterial strains in the living state. This was shown to be true for some strains of *Streptococcus pyogenes* and *Diplococcus pneumoniae* (Welsch, 1937*a*). A survey made in 1942, of the bacteriolytic properties of a large series of actinomycetes enabled us to state that about 20 per cent of the strains examined gave a filtrate capable of lysing, at least partly, suspensions of *S. aureus* (Welsch, 1942). Such active filtrates have been called actinomycetin (Welsch, 1937*b*), and, therefore, we feel justified in stating that actinomycetin is endowed with antibiotic activity.

It is true that we have found difficulties in demonstrating the activity of actinomycetin upon living cells, since this phenomenon is highly sensitive to a number of influences such as the pH of the medium, the concentration of mineral salts, the presence of peptones and carbohydrates, etc. However, it has been found recently that in culture media containing the right kind of peptone, for instance Difco tryptone or Wilson's bacteriological peptone, as well as a small amount of agar-agar (0.1 per cent) or 10 per cent starch or dextrin, the actinomycetin-producing strain "G" *Streptomyces albus* furnishes, after incubation in shallow layers at 25 C for 3 weeks, a paper filtrate which causes in 1 to 3 hours the lysis of living *S. aureus* suspended in water or in dilute buffer. This filtrate has also a lytic effect upon *Corynebacterium diphtheriae*, *Bacillus megatherium*, and *S. pyogenes*. The last organism may even be lysed by the filtrate when suspended in broth or peptone water. The antibiotic properties of actinomycetin are thus definitely established (Welsch, 1946).

Since actinomycetin is a crude culture filtrate, it cannot be stated that it is an antibiotic, but one may safely assert that it contains one or more antibiotic agents. It is possible that the lytic action of actinomycetin is the result of two types of activity: bactericidal action, followed by a secondary lytic effect upon the cells already killed by the first agent. This mechanism was assumed as a working hypothesis previously, when filtrates or concentrates active on living cells could not be obtained (Welsch, 1941). This hypothesis will have to be revised now since preparations of actinomycetin strongly active upon living cells can now be obtained. The results will be published shortly.

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THE VIABILITY OF HEAT-ACTIVATABLE SPORES IN NUTRIENT AND NONNUTRIENT SUBSTRATES AS INFLUENCED BY PRESTORAGE OR POSTSTORAGE HEATING AND OTHER FACTORS

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We have previously reported (Curran and Evans, 1945) that a large proportion of the spores of certain thermotolerant and thermophilic aerobes require a preliminary heat shock before they will germinate; in such cultures usually some of the spores do not require heat activation, but the majority will germinate only after they have been suitably activated. Since activation appears to initiate the germination process, it seemed reasonable to suppose that activated spores maintained under conditions inimical to completion of the germination process might lose their vitality rather rapidly. Subsequent observations proved the accuracy of this supposition and revealed that devitalization of heat-activated spores is causally connected with environmental conditions. The present paper deals with some of these conditions and indicates their relationship to viability in heat-activated spores.

METHODS AND MATERIALS

The organisms used and their sources were *Bacillus subtilis*, strains LB (Bureau of Dairy Industry), 6 (American Can Co.), FDA (Food and Drug Administration), 6634 (American Type Culture Collection); *Bacillus circulans*, 7049 (N. R. Smith); and thermophilic flat sour cultures, 1518 (National Canners Assn.), C_2P_3, C_1P_4, 07, M_{17} (American Can Co.). With two exceptions (6634 and 7049), all of the cultures were isolated from spoiled, commercially processed canned foods.

The spores were produced on standard beef extract tryptone agar and were incubated at the optimum temperature of the organism. The technique used in their collection and preparation has been described previously (Curran and Evans, 1945). The plate method of enumeration was employed; the plating medium was that used for the production of spores with 0.5 per cent glucose added. Plates were counted after 48 hours of incubation at temperatures optimal for the organism. The counts represent the averages of triplicate plates.

Heat treatments were conducted in a thermostatically controlled glycerol bath (±0.5 C). The buffers consisted of mixtures of 1 per cent K_2HPO_4 and KH_2PO_4 solutions in proportions suitable to give the desired pH values; all determinations of the latter were made with a Beckman glass electrode apparatus. Baker's cp glucose was used except where otherwise noted. Pyrex glassware was used throughout. After storage, sample tubes were closed with tightly fitting, sterile rubber stoppers and shaken for several minutes to ensure resuspension of the sedimented spores.

EXPERIMENTAL

In the first experiment, washed spores were suspended in sterile distilled water or 1 per cent glucose solution and thoroughly mixed. Aliquots of each were then dispensed into each of 4 sterile test tubes. Plate counts were made before and after heating at 95 C for 15 minutes, and again after storage at 37 C for varied periods with and without poststorage heating. The results are shown in table 1. Three of the cultures used were activated by heat; one, 6634, showed no direct response to heating but is included to furnish a basis for comparison. Neither the decrease of glucose to 0.1 per cent nor the substitution of an especially purified grade (U. S. Bureau of Standards) materially affected the recorded results.

TABLE 1

Viability of heat-activatable spores in distilled water and 1 per cent glucose solution as influenced by pre- or poststorage heating

(Storage temperature 37 C)

ORGANISM	DISTILLED WATER					1 PER CENT GLUCOSE SOLUTION			
	Time held	No heat	95 C—15 min before storage	95 C—15 min after storage	95 C—15 min before and after storage	No heat	95 C—15 min before storage	95 C—15 min after storage	95 C—15 min before and after storage
	days	*per ml*	*per ml*	*per ml*	*per ml*	*per ml*	*per ml*	*per ml*	*per ml*
B. subtilis (LB)	0	8,600	45,000	(45,000)	45,000	8,600	56,000	(56,000)	56,000
	2	8,000	23,000	45,000	33,000	6,000	170	44,000	420
	10	9,900	17,000	45,000	34,000	4,300	28	39,000	112
	30	10,400	18,600	39,400	37,900	3,800	8	33,000	72
B. subtilis (FDA)	0	4,800	84,000	(84,000)	93,000	4,900	88,000	(88,000)	103,000
	2	5,900	42,000	79,000	89,000	3,300	29,000	98,000	68,000
	10	5,000	19,000	75,000	71,000	2,200	15,000	72,000	46,000
	30	5,400	20,300	77,000	86,000	3,500	8,700	86,000	59,000
B. subtilis (6634)	0	106,000	109,000	(109,000)	109,000	115,000	97,000	(97,000)	97,000
	2	101,000	96,000	94,000	82,000	99,000	79,000	99,000	74,000
	10	96,000	91,000	86,000	80,000	86,000	81,000	86,000	75,000
	30	99,000	66,000	80,000	72,000	89,000	57,000	91,000	56,000
B. circulans	0	11,000	95,000	(95,000)	50,000	11,500	80,000	(80,000)	47,000
	2	9,900	440	80,000	8,900	7,600	2,080	73,000	8,100
	10	4,700	82	67,000	9,800	5,200	1,330	66,000	6,700
	30	5,000	134	57,000	10,300	4,500	2,750	53,000	8,500

( ) = potentially viable spores.

It is clear from these results (table 1) that the activation of spores by heat materially affects their subsequent viability when they are maintained under conditions unfavorable for their normal development. When the spores were activated and stored in distilled water, the number of cells which produced colonies decreased substantially during the first 10 days of storage. This loss of apparent viability was especially marked in *B. circulans*, with which the number of viable spores was reduced 99 per cent in 2 days. When the activatable spores were heated both before and after storage, the viable count was substantially increased over that in which only prestorage heating was employed. When the spores were heated after, but not before, storage, the count did not change greatly throughout the period of observation; likewise the spores receiving no heat either before or after storage underwent comparatively little change in the number of viable spores. The nonactivatable culture (6634) evidenced little change under similar conditions of manipulation. With this nonreactive culture, however, prestorage heating of the spores, though not affecting their capacity to germinate immediately, diminished appreciably their period of maximum viability.

When the spores were heated and stored in 1 per cent glucose solution, the losses in viability were greater and more rapid in the activatable *B. subtilis* cultures. The viability losses in *B. circulans* were, however, somewhat less in glucose than in water solutions. In both unheated suspensions and in those heated only after storage rather slow but progressive losses in viability occurred in the heat-activated spores on storage. It is of interest to observe that the culture showing greatest activation (*B. subtilis*, FDA) did not show the greatest or most rapid diminution in viability. Comparable results were obtained with culture 1518, a heat-activatable, flat sour type. Fluctuations in pH for LB were within the range 7.04 to 6.83, which indicates that acidity per se was not responsible for the observed mortality. The fact that poststorage heating substantially increased the plate count in those suspensions which received prestorage heating poses a question as to the cause of this reaction. It should be noted that even two initial heatings do not appreciably increase the count with the activatable *B. subtilis* cultures but reduce considerably the count of *B. circulans*.

Microscopical examination of hanging drop and stained (aqueous crystal violet) film preparations lead to the conclusion that some of the heat-activated spores, after pregerminative changes involving swelling and partial loss of refractility, deactivate—that is, revert to their original inactivated condition and thus require a second heating to condition them for germination. There was no indication that the increased count resulted from germination and resporulation. In the stained preparations the spores of the activatable *B. subtilis* cultures in both water and glucose solutions stained ringwise before, and similarly or slightly tinted throughout after, preliminary heating at 95 C for 15 minutes, and also throughout the period of rapid death (2 and 10 days). Similarly treated spores of *B. circulans* stained ringwise before initial heating, and before heating at 2 and 10 days, but stained solidly after heating before storage and after storage for 2 and 10 days.

It is apparent from the foregoing that spores activated and stored in certain nutritionally incomplete substrates may become nonviable with surprising rapidity. The described results were obtained in mediums approximately neutral and with storage at 37 C. The influence of these factors (pH and temperature) upon the mortality of stored heat-activated spores was next considered.

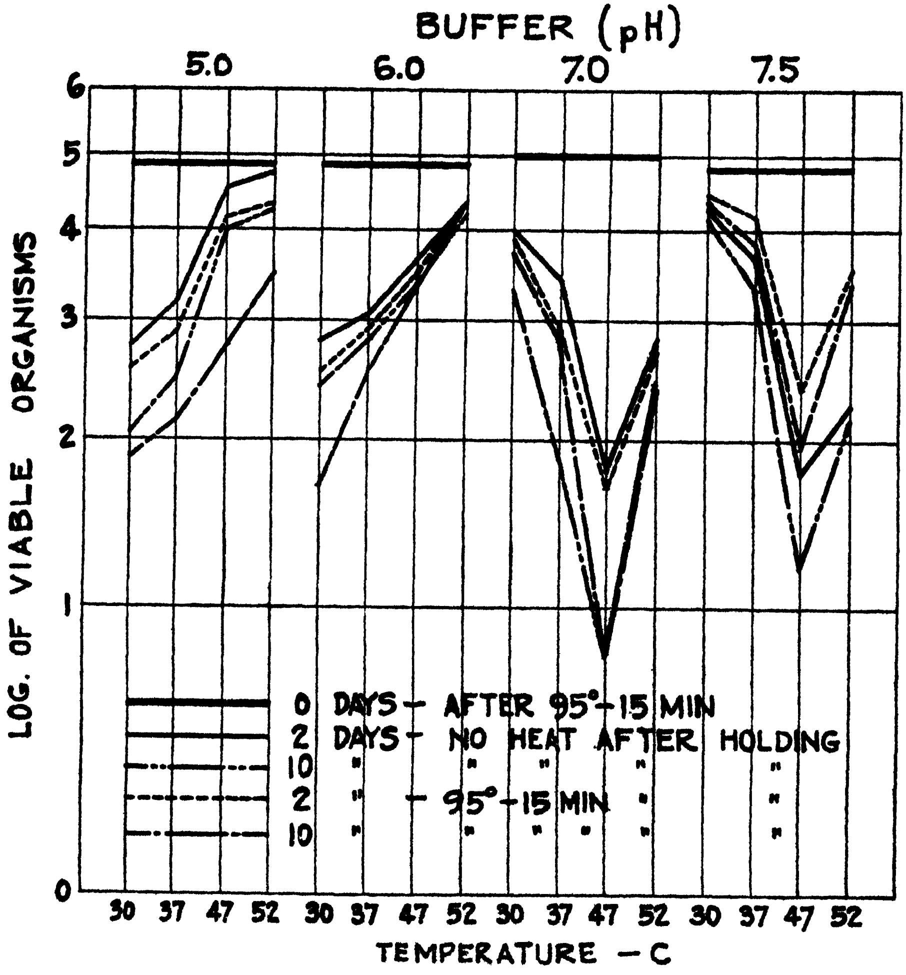

FIG. 1. THE INFLUENCE OF TEMPERATURE IN RELATION TO pH UPON THE VIABILITY IN STORAGE OF HEAT-ACTIVATED SPORES OF BACILLUS SUBTILIS LB

To accomplish this, phosphate buffer solutions containing 0.1 per cent glucose were so combined as to yield 4 buffer mixtures having pH values ranging from pH 5.0 to pH 7.5. Tubed sterile solutions of these buffer-glucose mixtures were seeded uniformly with diluted aqueous suspensions of washed spores, their reactions checked, and the suspensions heated at 95 C for 15 minutes, cooled, and stored at each of 4 temperatures. Plate counts were made initially (after

95 C for 15 minutes) and after 2 and 10 days of storage with and without poststorage heating. The results obtained with 3 activatable cultures are recorded in figures 1 to 3.

Each figure consists of a series of separate curves in which the logs of viable organisms (ordinates) are plotted against temperature (abscissae) in substrates of varying pH. The initial viable counts after activation at 95 C for 15 minutes are represented by the solid parallel lines. The curves show culture differences and also some areas of general agreement. Thus the counts after poststorage heating were generally higher than those not heated after storage; the margin of this difference in comparable samples is a measure of deactivation. This phenomenon was most pronounced at intermediate temperatures of storage (37 to 47 C), and usually greater in the neutral or alkaline substrates than in those definitely acid. Of interest also is the fact that with poststorage heating,

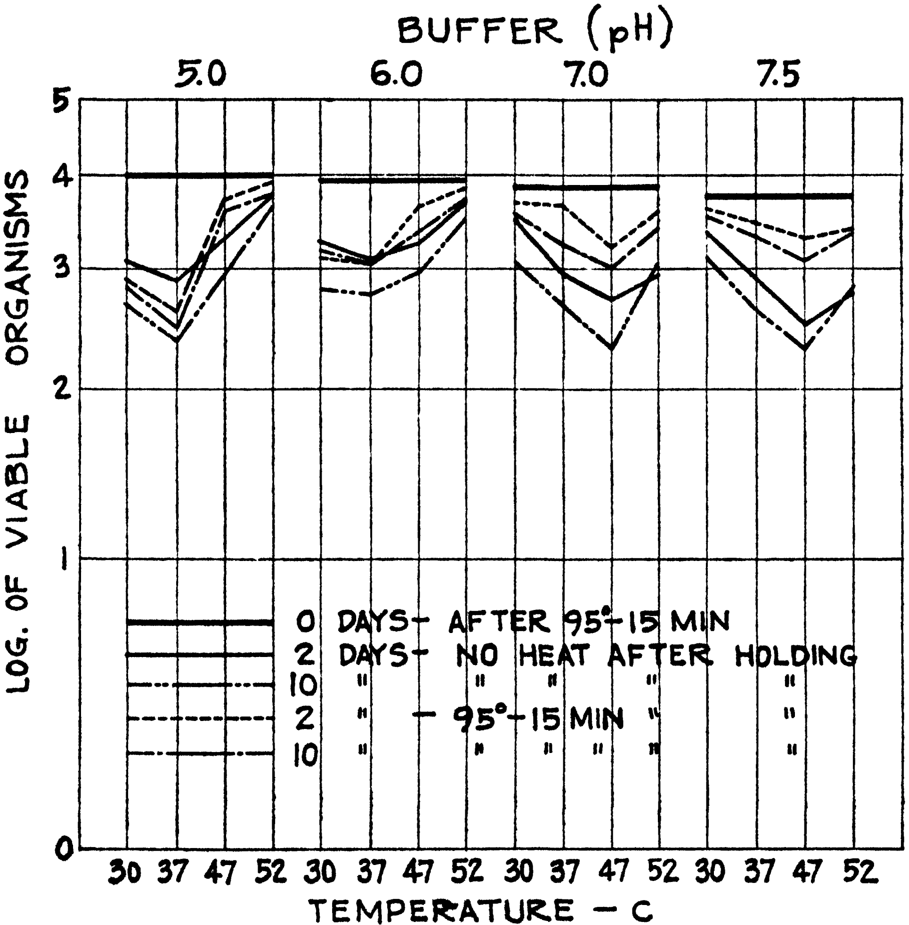

FIG. 2. THE INFLUENCE OF TEMPERATURE IN RELATION TO pH UPON THE VIABILITY IN STORAGE OF HEAT-ACTIVATED SPORES OF BACILLUS SUBTILIS FDA

the greatest mortality occurred not at the highest temperature of storage but at intermediate temperatures, as with deactivation. These intermediate temperatures approximate, in general, the optimum growth temperature of the organism. However, in *B. subtilis* LB in acid substrates devitalization was greatest at suboptimum growth temperature. In the two *B. subtilis* cultures minimum viability with or without poststorage heating occurred at a lower temperature in acid than in neutral or alkaline medium. Some observations were made upon samples subjected to fluctuating storage temperatures in the range of ±30 to 52 C. However, the losses in viability were in no instance more rapid or greater than those obtained under selected constant temperatures. In buffer suspensions, temperature-pH relationships are necessarily modified by a specific effect of the salt, although this factor is constant for any given pH.

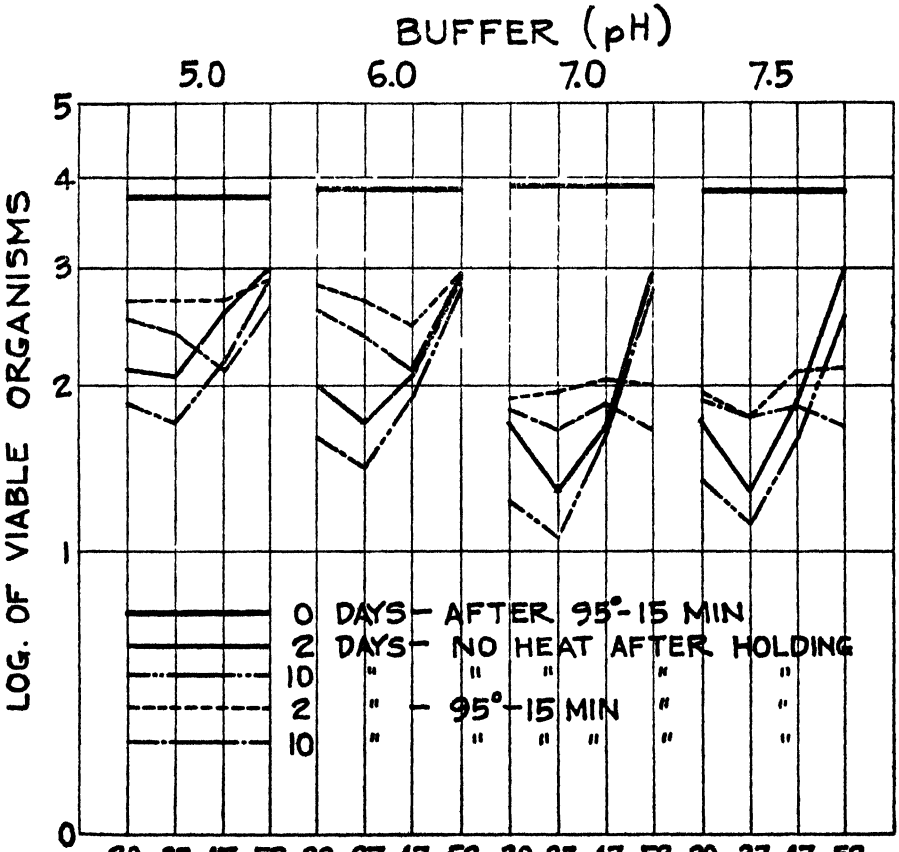

FIG. 3. THE INFLUENCE OF TEMPERATURE IN RELATION TO pH UPON THE VIABILITY IN STORAGE OF HEAT-ACTIVATED SPORES OF BACILLUS CIRCULANS

It has been shown that spores activated and stored in a nonnutritional or incomplete medium tend to lose viability rapidly with storage temperature within the normal range of growth of the organism. It next became of interest to study the behavior of spores activated and stored in a nutritionally complete medium but subsequently maintained at subminimum growth temperatures. In this instance, the block to complete germination becomes purely physical rather than physicochemical in nature. In table 2 are recorded the results obtained when the usual pre- and poststorage heating and storage were conducted in a milk substrate. The storage temperature for each culture was below that of normal germination and growth. The data (table 2) show that spores activated in milk and then stored at a temperature below that permissive of complete germination tend to lose their viability rather rapidly, though at a substantially slower rate than that which occurred in nutritionally incomplete mediums. The drop in count was greatest during the first month of storage, after which the rate of decline was very slow. There is very little evidence of deactivation in this medium; however, in a nutritionally complete substrate deactivation may be masked by a very gradual development of some of the spores with accompanying loss of heat stability.

TABLE 2

*The viability of heat-activated spores in milk as influenced by pre- and poststorage heatings**

ORGANISM	STORAGE		NO HEAT	PRESTORAGE HEAT ONLY	PRE- AND POST-STORAGE HEAT
	Period	Temperature			
	mo	*C*	*per ml*	*per ml*	*per ml*
B. subtilis	0		19,000	84,000	(84,000)
	1	3	21,000	53,000	35,000
	2	3	20,000	42,000	31,000
	4	3	24,000	27,200	24,600
C_2P_3	0		42,000	42,000	(42,000)
	1	16	46,000	12,100	
	2	16	44,000	9,900	8,700
	4	16		7,800	6,700
C_1P_4	0		45,000	46,000	(46,000)
	1	16	57,000	18,300	
	2	16	49,000	18,800	15,000
	4	16		14,700	13,900
07	0		54,000	70,000	(70,000)
	1	16	77,000	33,000	33,000
	2	16	62,000	36,600	30,800
	4	16		32,900	32,700
1518	0		62,000	66,000	(66,000)
	1	37	65,000	36,900	27,300
	2	37	73,000	24,300	21,100
	4	37		4,000	7,600

* 95 C for 15 minutes.

In table 3 are shown changes in the viability of spores in relation to storage after their exposure to severe heating in milk—the heating being sufficient to kill all but a very small proportion of the initial population. As may be seen, the highly resistant fraction of the spores of *B. subtilis* LB underwent little change in viability; the surviving flat sour spores in contrast died rather rapidly with storage. Wheaton and his associates have observed a similar destruction of thermophilic flat sour spores in creamed corn when this product was inoculated before processing and subsequently stored at subminimum growth temperatures;

TABLE 3

The viability of spores in milk after heating at 115 C without poststorage heating at 95 C for 15 minutes

ORGANISM	INITIAL CONCENTRATION	HEATED AT 115 C	STORAGE		VIABLE SPORES
			Period	Temperature	
	per ml	*min*	*mo*	*C*	*per ml*
B. subtilis LB	121,000	20	0		20,900
			1	3	33,100
			2	3	36,400
			4	3	32,000
	121,000	32	0		0.6
			1	3	0.6
			2	3	0.3
			4	3	0.3
C_2P_8	250,000	15	0		2,570
			1	16	260
			2	16	108
			3	16	44 (7)
C_1P_4	234,000	15	0		1,280
			1	16	380
			2	16	270
			3	16	248 (173)
07	334,000	20	0		370
			1	16	8.6
			2	16	9.0
			3	16	0.6 (2.6)
M 17	49,000	20	0		710
			1	16	690
			2	16	360
			3	16	150 (43)
1518	900,000	60	0		620
			1	16	4.3
			2	16	1
			3	16	0.6 (0)

( ) = after heating at 95 C for 15 minutes.

complete sterility was attained in about 6 months (unpublished). The foregoing observations suggest possibilities for the elimination of flat sour spores by manipulation of heat treatment and storage temperatures.

DISCUSSION

Heat activation in bacterial spores has its counterpart in certain of the fungi, notably in the ascospores of *Ascobolus* and *Neurospora* (Dodge, 1912; Shear and Dodge, 1927; Goddard, 1935) and in *Phycomyces* (Robbins, Kavanagh, and Kavanagh, 1942). The process of activation in *Neurospora tetrasperma* (Goddard, 1935) was shown to be completely reversible (deactivatable). Since activation in bacterial spores has been observed only in certain aerobic species, a relationship between aerobic sporeformers and the true fungi is suggested. Similarities between the sporeforming bacilli and the *Mucorini* and other fungi have been pointed out by Cook (1932).

The observed effects of heat activation upon the viability of spores and the influence thereon of various physical and chemical factors encourage speculation as to the nature of the devitalizing process. In nutritionally incomplete substrates providing a utilizable carbohydrate, the development of sufficient acidity might conceivably induce a rapid death of the activated spores, but with *B. subtilis* LB in glucose solution, over 99 per cent of the spores became rapidly nonviable without appreciable change in reaction. Phage virus is transmissible by spores (Dooren de Jong, 1931; Cowles, 1931; Adant, 1932) and presumably may attack spores; however, there was no evidence that lysis of the spores occurred in our experiments.

The rapid mortality of spores following their activation and storage in water or glucose solutions and the slower mortality rate of similarly treated spores in milk suggest the presence in milk of a substance which protects the organisms from rapid lethal action. In this connection the findings of Winslow and Brooke (1927) appear to have some relevance. These authors found that young vegetative cells of *Bacillus cereus* and *Bacillus megatherium* died with extreme rapidity when suspended in distilled water and centrifuged twice to remove substances carried over from the agar slope on which they had previously been grown. A high degree of protection was afforded by extreme dilutions of nutrient broth, or its component ingredients, but not by sugar or balanced salt solutions. We may assume that heat activation provides a definite impulse toward germination; concomitant with or soon after activation spores become perceptibly less sporelike and more like vegetative cells. It might be reasoned, therefore, that heat-activated spores become in effect very young vegetative cells and in consequence react in pure water and glucose solutions as did the vegetative cells of the sporeformers described by Winslow and Brooke (1927). However, this interpretation is contraindicated, first because in both *B. subtilis* cultures rapid losses in viability were not accompanied by greatly increased permeability of the spores to crystal violet—a change which occurs in the process of normal germination and which is associated with or preceded by a material decrease in heat stability. Moreover, young vegetative cells of *B. subtilis* and *B. circulans* have not in our hands shown susceptibility to pure distilled water and glucose solutions comparable in degree to that described for *B. cereus* and *B. megatherium.*

In the spores of *Phycomyces*, heat activation seems to effect a diffusion of essential growth substances from the cells into the surrounding medium (Robbins *et al.*, 1942). Whether this reaction is in any way responsible for the devitilization of such spores is not known, nor is it yet known whether a similar leaching out of cell contents occurs following the heat activation of bacterial spores. This point will be the subject of further investigation.

It might be postulated that spores as a result of activation are stimulated to intense metabolic activity. In the absence of an extraneous source of nitrogen or energy, the contained supply of these essential food elements would be rapidly exhausted. If the nitrogen-containing substances are depleted and a source of energy, either intra- or extracellular, is still available, a situation may be conceived in which catabolic processes gain the ascendancy and lead to cell death from internal causes. An enzymic reaction of this nature might be expected to have rather definite temperature optima varying with environmental conditions. By this hypothesis glucose or other utilizable carbohydrate would greatly *accelerate* the death process of activated spores in an incomplete medium, provided an excess of available energy was not present in the cells, and would have no such effect if sources of energy were available within the cell. The reaction with *B. subtilis* LB accords with the first condition, that with *B. circulans* with the second. Knaysi (1945) demonstrated considerable nitrogen-containing reserve material in the spores of a culture of *Bacillus mycoides* but found no utilizable sources of energy. Just why the activation process is reversible for some spores under particular conditions (deactivation), yet for other individuals under similar conditions is irreversible, is not apparent. In earlier observations on heat activation of spores in water and glucose solutions (Curran and Evans, 1945) we found no evidence for deactivation, probably because of the very low storage temperature (3 C).

SUMMARY

The spores of aerobic, mesophilic and thermophilic, heat-activatable cultures undergo profound and rapid alteration in viability when activated and stored under conditions unfavorable for complete germination.

When activated and stored in nutritionally incomplete substrates (distilled water or glucose solution), spores die rapidly or undergo deactivation with retention of viability; when activation is conducted in a nutritionally complete medium (milk) with storage at subminimum growth temperatures, the spores die more slowly and there is little evidence of deactivation. Unheated, activatable or nonactivatable spores undergo little or no change in viability over long periods; in glucose solution the rate of deterioration is measurably increased.

The reaction associated with the rapid death of heat-activated spores has a temperature optimum ranging with the organisms studied from 30 to 47 C, depending upon the organism and the pH of the substrate.

A theory for the mechanism of the observed reactions is advanced.

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THE EFFECT OF CERTAIN MINERAL ELEMENTS ON THE PRODUCTION OF PENICILLIN IN SHAKE FLASKS[1, 2]

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In earlier publications from these laboratories (Knight and Frazier, 1945*b*; Koffler, Knight, Frazier, and Burris, 1946) attention was directed to the fact that the addition of corn steep ash to certain synthetic media significantly increased the production of penicillin as well as the rate of other metabolic reactions. Although the importance of mineral elements in the production of penicillin by submerged mold cultures has been emphasized by Foster, Woodruff, and McDaniel (1946), Moyer and Coghill (1946), and Pratt and Hok (1946), no suggestions were offered as to which constituents of the corn steep ash were responsible for the higher penicillin levels in synthetic media. This paper attempts to answer that question.

METHODS

Penicillin was produced by submerged cultures of *Penicillium chrysogenum* X-1612 (agitated in a reciprocating shaker) in the manner outlined by Koffler, Emerson, Perlman, and Burris (1945). The following basal medium was used:

Lactose, U.S.P.	25.00 g
Starch	5.00 g
Dextrin, N.F.V.	5.00 g
Glacial acetic acid, cp	6.00 g
$(NH_4)_2SO_4$, reagent	5.00 g
KH_2PO_4, reagent	1.50 g
$MgSO_4 \cdot 7H_2O$, U.S.P.	0.25 g
$ZnSO_4 \cdot 7H_2O$, reagent	0.04 g

Distilled water to 1 L
pH adjusted to 6.3 with KOH before autoclaving
Supplements made and constituents removed, as indicated in the text, before autoclaving

This basal medium was only slightly different from the one used previously (Knight and Frazier, 1945*b*) and without supplements permitted excellent growth of the mold but no appreciable penicillin yields. Because this suggested that metallic contaminants, if they occurred at all, were not present in high enough concentration to affect penicillin production, it did not seem necessary to purify

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[2] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

the medium. The medium used for the growth of the spores has been described by Knight and Frazier (1945*a*).

Corn steep ash was obtained by heating corn steep solids (University of Wisconsin no. 71, purchased on July 20, 1945, from the Staley Company, Decatur, Illinois) in an electric furnace at 760 C for 4 to 5 hours. No attempt was made to dissolve the ash before it was added to the medium.

Penicillin assays were made by the Oxford cup method with *Staphylococcus aureus* FDA209P as the test organism (Foster and Woodruff, 1944; Schmidt and Moyer, 1944). A penicillin preparation from the Food and Drug Administration served as the reference standard. The method of Fiske and SubbaRow (1925) was used for the determination of phosphorus, and the method of Saywell and Cunningham (1937) was used for the determination of iron.

EXPERIMENTAL

Subsequent to the demonstration by Knight and Frazier (1945*b*) that the addition of corn steep ash to a synthetic medium increased penicillin yields, experiments were designed to determine which ash constituent or combination of constituents was responsible for this stimulation. The general procedure was to study the chemical composition of the ash and then observe the effect of various ash components, added both singly and in pairs, on the accumulation of penicillin in the basal medium.

The chemical composition of the corn steep ash. Knight and Frazier (1945*b*) found that 500 mg of corn steep ash was required for maximum penicillin production in 100 ml of synthetic medium.[3] The following method was used to prepare soluble and insoluble fractions of the whole ash and to determine their effect on penicillin yields: Simulating the conditions during the preparation of the fermentation medium, 500 mg of ash were put into 100 ml of water, and the pH was adjusted to 6.3 with glacial acetic acid. After autoclaving for 15 minutes at 15 pounds, 213 mg (42.6 per cent) of ash were in solution while 287 mg (57.4 per cent) remained as granular sediment on the bottom of the flask.

Table 1 indicates that the ability of the ash to stimulate the biosynthesis of penicillin resided almost entirely in its insoluble fraction. The soluble portion was slightly stimulatory, but both fractions were necessary for maximum penicillin yields.

Table 2 presents a qualitative spectrographic analysis of the whole ash, and of its soluble and insoluble fractions.[4] The following metals have not been detected: Sb, Be, Bi, Cd, Cb, Ge, Au, La, Hg, Pt, Sr, Ta, Ti, V, and Zr.

The stimulatory action of compounds, added singly and in combinations, on penicillin production in the basal medium. Salts of all the elements listed in table 2, with the exception of Si, were added in various amounts to the basal medium, but none increased penicillin yields as markedly as did corn steep ash. Details of some of these experiments were presented by Knight (1946).

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[3] This was approximately equivalent to the amount of ash in 3 per cent corn steep solids.

[4] We are grateful to Dr. Y. SubbaRow of the Lederle Laboratories and Mr. W. L. Dutton of the American Cyanamid Company for furnishing us with this analysis.

Since preliminary experiments indicated that combinations of Fe salts and phosphates enhanced penicillin yields as greatly as did corn steep ash, quantitative analyses for Fe and P were made on the whole ash and its two fractions. It was found that the whole ash, the soluble ash, and the insoluble ash contained P in concentrations of 12.9 per cent, 5.3 per cent, and 18.3 per cent, respectively, and Fe in concentrations of 0.27 per cent, 0.01 per cent, and 0.47 per cent, respectively.

TABLE 1

The effect of corn steep ash and of two ash fractions on the production of penicillin by Penicillium chrysogenum X-1612 in the basal medium

(Each figure is the average of three experiments)

BASAL MEDIUM PLUS (MG/100 ML)			PENICILLIN (OXFORD UNITS/ML)				
			Days				
Whole ash*	Soluble ash	Insoluble ash	4	5	6	7	8
			18	29	39	35	9
500			88	110	151	132	127
	213		32	48	57	75	62
		287	69	100	141	121	124
	213	287	91	106	152	118	125

* Five hundred mg of whole ash contained 213 mg of soluble constituents and 287 mg of insoluble constituents.

TABLE 2

Spectrographic analysis of corn steep ash and of two ash fractions

The ranges for qualitative estimates are as follows:

8 is 100 to 1%
7 is 10 to 0.1%
6 is 1.0 to 0.01%
5 is 0.1 to 0.001%
4 is 100 to 1.0 ppm
3 is 10 to 0.10 ppm
2 is 1.0 to 0.01 ppm
1 is less than 0.1 ppm
x is metal not detected
A is whole ash
B is soluble ash
C is insoluble ash

ELEMENT	A	B	C	ELEMENT	A	B	C
Al	3	3	2	Mg	8	4	8
As	4	4	4	Mn	6	4	6
B	6	5	6	Ni	4	3	4
Ca	7	7	7	P	8	5	8
Cr	6	3	6	K	8	8	7
Co	2	x	2	Si	7	7−	6
Cu	6	4	6	Ag	1	1	1
Fe	7	7	6	Sn	2	2	2
Pb	5	4	4	W	5	5	5
Li	2	2	1	Zn	5	2	4

Table 3 gives the penicillin yields when the basal medium was supplemented with either corn steep ash, or Fe and P in concentrations in which these elements occur in corn steep ash. The insoluble P was added arbitrarily as $Ca_3(PO_4)_2$ (reagent grade), and the soluble phosphates were added as KH_2PO_4 (reagent grade). The stimulatory effect of Fe salts did not depend upon their relative solubility (for example, $Fe_2(SO_4)_3$ showed the same effect as an equivalent amount of $FePO_4 \cdot 2H_2O$), and Fe was usually supplied as $Fe_2(SO_4)_3$, although in the ash Fe was present almost entirely in an insoluble form.

Table 3 reveals that the addition of both Fe salts and phosphates to the basal medium raised penicillin yields as markedly as did the addition of corn steep ash. Fe was highly stimulatory by itself, but not to the same degree as was whole steep ash; in the absence of Fe, phosphates showed scarcely any effect on penicillin production. Media containing both Fe and soluble phosphates supported penicillin production as well as did media with corn steep ash.

TABLE 3

The effect of corn steep ash, soluble P, insoluble P, and Fe on the production of penicillin by Penicillium chrysogenum X-1612 in the basal medium

(Each figure is the average of three experiments)

BASAL MEDIUM PLUS (MG/100 ML)				PENICILLIN (OXFORD UNITS/ML)				
				Days				
Ash	Sol. P*	Insol. P†	Fe‡	4	5	6	7	8
				18	29	39	35	9
500				88	110	151	132	127
		50		17	23	32	37	32
	10			22	36	41	32	27
			1.4	75	101	118	138	129
	10	50		19	35	52	55	53
		50	1.4	62	105	138	112	119
	10		1.4	73	108	149	125	124
	10	50	1.4	71	102	142	115	120

The Fe and P content of 287 mg insoluble ash was 1.35 mg and 52.5 mg, respectively, and of 213 mg soluble ash, 0.02 mg and 11.3 mg, respectively.

* As KH_2PO_4.

† As $Ca_3(PO_4)_2$.

‡ As $Fe_2(SO_4)_3$.

Table 4 corroborates the contention that the physiological function of corn steep ash can be attributed mainly to Fe and soluble phosphates. Insoluble ash (rich in Fe and insoluble phosphates but free from soluble phosphates) plus soluble phosphates enhanced penicillin production as greatly as did whole ash. The addition of soluble corn steep ash (rich in soluble phosphates but containing merely traces of Fe) caused maximum penicillin yields only when Fe was present in the medium.

The fact that corn steep ash contains a large number of constituents suggested that good penicillin yields might also be obtained in a simplified synthetic medium consisting of lactose, starch, dextrin, glacial acetic acid, $(NH_4)_2SO_4$, and ashed corn steep. Experiments indicated that this assumption was justified. Corn steep ash—and any contaminants which might have been present as traces in the other components of the medium—satisfactorily served as a source of the mineral elements essential to maximum penicillin formation.

The importance of ion antagonism in the production of penicillin. It was observed that the addition of 0.2 mg Cu to 100 ml of the basal medium completely inhibited penicillin production without visibly affecting the growth of the mold. The addition of only 0.1 mg of Fe to such a fermentation minimized the "toxic" effect of Cu, and higher amounts of Fe neutralized the inhibition completely. Table 5, which contains the results of a few typical experiments, strikingly illustrates this relationship.

TABLE 4

The effect of soluble and insoluble corn steep ash on the production of penicillin by Penicillium chrysogenum X-1612 in the basal medium which was also supplemented with Fe or phosphates, or both

(Each figure is the average of three experiments)

BASAL MEDIUM PLUS (MG/FLASK)					PENICILLIN (OXFORD UNITS/ML)				
					Days				
Sol. ash	Sol. P*	Insol. ash.	Insol. P†	Fe‡	4	5	6	7	8
	10	287			92	112	147	128	118
213				1.4	75	105	145	126	112
213			50		23	41	55	62	52
213			50	1.4	78	102	143	134	128

* As KH_2PO_4.
† As $Ca_3(PO_4)_2$.
‡ As $Fe_2(SO_4)_3$.

The question arose whether this "antagonism," which found its expression in *yields* of penicillin, affected the *biosynthesis* or merely the *stability* of penicillin. The effect of Fe or Cu, or both, on the stability of penicillin was observed as follows: Enough commercial penicillin (Pfizer)[6] was dissolved in a K_2HPO_4—KH_2PO_4 buffer of the desired pH (5.8, 6.4, 7.0, and 7.6) to give a concentration of approximately 120 Oxford units per ml. One-hundred-ml portions of this solution were placed in 500-ml Erlenmeyer flasks, supplemented as indicated in table 6, and agitated by a reciprocating shaker under the same conditions as were shake flask cultures. The penicillin content of the solution was assayed at the start and after 8, 24, and 48 hours. Table 6 demonstrates that Cu decreased the stability of penicillin solutions; at pH 5.8 Fe appeared destructive. At pH values of 6.4, 7.0, and 7.6 the action of Cu was offset by Fe.

From these experiments it would appear as if the association between penicillin yields and Fe-Cu antagonism could be explained more satisfactorily by strictly chemical (i.e., penicillin destruction) than physiological (i.e., penicillin synthesis) reactions. Such an interpretation, however, would be based on the assumption that penicillin responds to the action of Fe and Cu in actual fermentation liquors as it did in phosphate buffer. This was tested in the following manner: *P. chrysogenum* X-1612 was grown in shake flasks on the basal medium (containing no added Fe or Cu) for 4 days; then the liquor was freed of pellets and sterilized by filtration through a Berkefeld filter. Commercial penicillin was dissolved in this sterile solution to give a concentration of 140 Oxford units per ml. After the addition of Fe or Cu, or both, aliquots were shaken as they had been in the previous experiment. Penicillin assays were done at the start, and after 8, 24, 48, and 76 hours. Table 7 shows that in the presence of organic matter, at pH

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[6] Approximately 75 per cent of this sample consisted of penicillin G and 25 per cent was penicillin K. We are indebted to Mr. K. Higuchi for doing the differential assay.

TABLE 5

The effect of Fe and Cu on the production of penicillin by Penicillium chrysogenum X-1612 in the basal medium

(Each figure is the average of three experiments)

BASAL MEDIUM PLUS (MG/100 ML)		PENICILLIN (OXFORD UNITS/ML)			
		Days			
Fe ($Fe_2(SO_4)_3$)	Cu ($CuSO_4 \cdot 7H_2O$)	4	5	6	7
0	0	40	23	16	10
0	0.01	43	21	16	8
0	0.05	30	15	5	0
0	0.2	0	0	0	0
0	1.0	0	0	0	0
0.1	0	81	89	94	90
0.5	0	85	97	123	118
2.0	0	88	100	122	119
10.0	0	83	90	103	109
0.1	0.01	83	90	92	91
0.5	0.01	84	99	125	115
2.0	0.01	83	96	121	109
10.0	0.01	83	86	97	110
0.1	0.05	79	69	58	42
0.5	0.05	73	96	119	121
2.0	0.05	80	95	121	123
10.0	0.05	83	89	108	118
0.1	0.2	12	13	15	0
0.5	0.2	63	42	36	28
2.0	0.2	89	108	132	121
10.0	0.2	76	95	109	115
0.1	1.0	0	0	0	0
0.5	1.0	28	32	84	96
2.0	1.0	80	88	92	90
10.0	1.0	79	102	113	123

7.0, neither Cu nor Fe affected the stability of commercial penicillin. This would justify the assumption that the interaction between Fe and Cu was more likely connected with the formation than with the destruction of penicillin.

TABLE 6

The effect of Fe and Cu on the stability of penicillin in phosphate buffer at various hydrogen ion concentrations and 24 C

pH	BUFFER PLUS (MG/100 ML)		PENICILLIN (OXFORD UNITS/ML)			
	Fe ($Fe_2(SO_4)_3$)	Cu ($CuSO_4 \cdot 5H_2O$)	Hours			
			0	8	24	48
5.8			118	113	90	99
	10.0		118	96	45	26
		0.5	118	66	35	0
	10.0	0.5	118	67	20	0
6.3			120	123	86	88
	10.0		120	130	100	118
		0.5	120	56	20	15
	10.0	0.5	120	113	75	83
7.0			124	118	93	98
	10.0		124	118	96	90
		0.5	124	53	27	12
	10.0	0.5	124	123	75	79
7.6			120	103	95	81
	10.0		120	106	90	100
		0.5	120	45	0	0
	10.0	0.5	120	110	75	70

TABLE 7

The effect of Fe and Cu on the stability of penicillin in the fermentation liquor at pH 7 and 24 C

FERMENTATION LIQUOR PLUS (MG/100 ML)		PENICILLIN (OXFORD UNITS/ML)				
Fe ($Fe_2(SO_4)_3$)	Cu ($CuSO_4$ $5H_2O$)	Hours				
		0	8	24	48	72
		144	144	140	142	132
	1.5	140	140	140	138	134
30.0		140	138	134	148	134
30.0	1.5	144	138	150	140	130

DISCUSSION

This paper has attempted to show that the increase in penicillin yields which was observed when corn steep ash was added to a basal medium was actually due to the Fe and phosphates in the ash. Considering the great number of minerals in the ash, the difficulty of such an attempt is apparent. Combinations of elements other than Fe and P might eventually be shown to be as stimulatory as was corn steep ash. In fact, unpublished experiments indicate that combinations of Fe and Cr raised penicillin yields almost as much as did Fe and phosphates. Pratt and Dufrenoy (1945) must have noted the stimulating effect of Cr because they included this element in their synthetic medium.

Since media low in Fe never gave high penicillin yields, Fe can be regarded as the most indispensable constituent of corn steep ash. To obtain maximum penicillin production, however, the addition of both Fe and soluble phosphates was prerequisite. It should be noted that the level of Fe and soluble phosphates necessary for maximum penicillin production was greater than the level for maximum growth of the mold.

The function of Fe and P in the production of penicillin is unknown. These elements may either affect the formation or the stability of penicillin. Many enzymes carry Fe in their prosthetic groups, and such enzymes may be involved in the biosynthesis of penicillin. On the other hand, Fe may appear necessary in penicillin production because it protects penicillin from the destruction catalyzed by other elements. Hutner (1946) holds the general view that many elements are considered essential nutrients because they form precipitates in dilute media and thereby remove toxic elements by precipitation or adsorption. However, there is no reason to assume that Fe affected penicillin production in this manner because the basal medium that was employed in these studies already contained high levels of starch and dextrin, which are able to act as adsorbents.

It is even more difficult to explain the role of soluble phosphates, since KH_2PO_4 was present in the medium and increments of P neither caused additional mold growth nor changed the pH picture of the fermentation. Pulvertaft and Yudkin (1945) found it possible to stabilize penicillin solutions by the addition of phosphates, a protection which was shown not to be due to an effect of pH. The degree of stabilization depended upon the sample of penicillin, the concentration of penicillin in the solution, and the concentration of phosphate. Different samples differed in the amount of phosphate that gave maximal protection. The enhancement of penicillin yields by soluble phosphates in the presence of Fe might be explained, at least tentatively, in the light of Pulvertaft and Yudkin's experiments.

The observation that Cu impaired penicillin production but that Fe was able to offset this damage may be of practical as well as theoretical importance. Industrially, corn steeps with unusually high contents of Cu might be improved by supplements of Fe. Theoretically, a study of the counteraction between Fe and Cu might help to elucidate the mechanism of penicillin synthesis. Of course, such a study would be valuable only if Cu actually interfered with penicillin formation and not with the stability of penicillin already synthesized. The fact that Cu did not destroy commercial penicillin that had been added to fermentation liquors suggests that the interplay between Fe and Cu influenced more fundamental reactions than the destruction of penicillin.

It is probable that antagonistic relationships between elements other than Fe and Cu will be demonstrated. An incomplete survey made in this laboratory has furnished evidence that Fe can also prevent the destruction of penicillin by Al.

It was realized that the inorganic constituents of the medium may affect not only the quantity of penicillin but also the type of penicillin produced; a study of this problem, however, was beyond the scope of this investigation.

SUMMARY

The ability of corn steep ash to increase penicillin production by *Penicillium chrysogenum* X-1612 in a basal synthetic medium was reproduced by the addition of Fe and soluble phosphates.

The presence of copper (>2 ppm) in the basal medium completely prevented the accumulation of penicillin; the addition of only 1 ppm of iron offset the effect of copper. Evidence indicated that this interaction between iron and copper affected the synthesis rather than the destruction of penicillin.

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NOTE

NUTRITIONAL REQUIREMENTS OF STREPTOCOCCUS APIS[1]

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Although *Streptococcus apis* is considered identical with *Streptococcus liquefaciens* (Davis and Tarr: Nature, **138,** 763), the nutritional requirements of which were reported recently (Niven and Sherman: J. Bact., **47,** 335), strains of the former were included in a general study of the vitamin and amino acid requirements of bacteria associated with foul brood diseases of bees to determine whether this organism as isolated from bee larvae differed in growth requirements from those isolated from human and other sources. Two stock strains (239 and 395) were available for study. The procedure and reagents used are described in another paper (Katznelson and Lochhead: J. Bact., **53,** 83).

Both cultures were found to require pantothenic acid, nicotinic acid, pyridoxine, and biotin; one required folic acid and was stimulated by thiamine; but neither required riboflavin. Purine and pyrimidine bases exerted no effect. Thus with respect to the first four vitamins these cultures are similar to the enterococci studied by Niven and Sherman. However, the *S. liquefaciens* strains used by these workers evidently required riboflavin and grew in the absence of folic acid and thiamine. The enterococci in general were stimulated by purine and pyrimidine bases.

The amino acid requirements of the *S. liquefaciens* and *S. apis* strains could not be compared directly, as the data supplied by Niven and Sherman applied specifically to one strain of *Streptococcus zymogenes*. Both *S. apis* strains grew in a medium containing 14 amino acids: glycine, valine, leucine, aspartic acid, glutamic acid, cystine, histidine, lysine, arginine, proline, isoleucine, methionine, asparagine, and tryptophane. Of these, valine, leucine, and glutamic acid were essential for both; histidine, arginine, and tryptophane were essential for strain 239; and the remainder, particularly glycine and cystine, were stimulatory. Nine of these acids are included in the list of 13 given by Niven and Sherman. Ammonium sulfate when added to the essential acids stimulated strain 395 but inhibited strain 239. Both strains grew upon continued subculture in the vitamin amino acid medium.

It appears that certain distinct differences exist between these two strains of *S. apis* and the *S. liquefaciens* strains studied by Niven and Sherman with respect to both vitamin and amino acid requirements. However, strain variation among the enterococci is apparently very wide and may embrace such differences as have been observed in nutritional needs.

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[2] Agricultural scientist.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

NEW YORK CITY BRANCH

Columbia University College of Physicians and Surgeons,
New York, October 22, 1946

Testing of Fungicidal Materials against Pathogenic Fungi. *Louis C. Barail*, United States Testing Company, Hoboken.

As there is no official method for the testing of antiseptic compounds or treated materials, the author has studied comparatively the existing methods and here describes their advantages and imperfections. None of them appears to be suitable to the great variety of chemicals and materials that have to be tested against pathogenic fungi. Among such materials are animal and vegetable fibers, leather, finishes, paints, lacquers, plastics, cutting and quenching oils, soaps, cosmetics, and many others.

A recontamination method is described, which permits reproducible and accurate results. All these materials can be tested, and the organisms used are fresh standard cultures of fungi of the *Tricophyton*, *Microsporum*, *Achorion*, or *Epidermophyton* groups, and also *Monila albicans*. The method can also be used advantageously to replace the old phenol coefficient test, which is not applicable to fungi. Safety tests are also being conducted on all samples to ascertain that they are nontoxic to man, nonirritating, and nonsensitizing to human skin.

Of more than 300 fungicides so tested, very few have proved to be satisfactory. The best results for nontoxicity and harmlessness to human skin as well as efficiency against most pathogenic fungi were obtained with a few long-chain organic mercury compounds.

Observations on the Stability of Certain Bacteriophages. *Morris L. Rakiten*, Long Island College of Medicine, Brooklyn.

With two exceptions, all of the bacteriophages had been produced in a "savita" broth medium. The exceptions, coli phages B1 and B2, had been prepared in a peptone meat extract broth. Sealed ampoules of the phages (2 ml) had been stored in the ice chest (2 to 5 C) from 7 to 17 years. The strains of bacteria originally used for the propagation of the coli phages and the single race of subtilis phage were available. For the staphylococcus phages, freshly isolated strains were employed.

Coli phages B1 and B2 had been saved since 1929. B1 is viable but is greatly attenuated; repeated passages do not increase its corpuscular count. Of three coli phages stored for 10 and 11 years, one is inactive. One of the other two is active in dilutions of 10^6, the second at 10^2. After two passages, lysis results in dilutions of 10^{10}. The subtilis phage stored for 12 years is inactive. Four polyvalent staphylococcus phages stored for 11 and 13 years are inactive. One staphylococcus phage 7 years old is active, and following five passages against a susceptible strain is polyvalent, lysing cultures in dilutions above 10^7.

Studies of Streptomycin on Salmonella Cultures. *Erich Seligmann and Michael Wassermann*, Beth Israel Hospital, New York.

With a serial broth dilution method and a suitable colon bacterium, 226 *Salmonella* strains, representing 60 different types, were tested and found sensitive to streptomycin. Most strains yielded to 4 to 8 units. Mouse experiments with oral infection by *Salmonella typhi-murium*, *Salmonella enteritidis*, *Salmonella cholera-suis*, *et al.*, and oral or oral and subcutaneous treatment resulted in the suppression of the normal fecal flora and the pathogens. After termination of the treatment the fecal flora and *Salmonella* organisms reappeared. The death rate was similar to that of untreated controls, although the time of survival was prolonged.

In *Salmonella* enteritis of infants the same streptomycin effect was observed: temporary suppression of pathogens and fecal flora, and reappearance after discontinuation of treatment.

Studies on the varying resistance of strains revealed that most cultures were not a biological entity, but were composed of bacterial cells of different streptomycin sensitivity. With E. Fishberg's collaboration it was found that the reducing power of growing organisms had a relationship to their streptomycin sensitivity. The higher the activity, the higher the resistance to streptomycin. Thus an approach is opened to the chemical determination of streptomycin sensitivity of bacterial strains.

STREPTOMYCIN IN THE TREATMENT OF PYOCYANEUS INFECTIONS OF THE URINARY TRACT. *Lewis H. Schwarz and Joseph A. Lazarus,* New York.

This study deals with the effectiveness of streptomycin in the treatment of urinary tract infections due to various strains of *Pseudomonas aeruginosa.* Experiments were conducted upon eight patients with infections involving the kidneys, bladder, and blood stream. In two instances, the organism was of the mucoid variety. Susceptibility to streptomycin varied from 12.5 to 250 S units. The greatest resistance to the antibiotic was shown by the mucoid variant. Of the six instances in which the infection was due to nonencapsulated organisms, there were two cures (one with a positive blood culture). One patient with suppurative pyelonephritis due to the mucoid variant of *P. aeruginosa* was cured only after the removal of the involved kidney. In the cured cases, the first negative culture was obtained only after the administration of 8 million units of streptomycin, full effectiveness requiring 13 to 20 million units.

Our experiments showed that the endotoxin was solely responsible for the toxicity. In the case of the mucoid variety of organism, streptomycin caused a mutation of the organism to the rough phase, with a concomitant reduction in virulence. Following cessation of treatment, a reversal of the process occurred with full restitution of virulence.

THE AGGLUTINATIVE REACTION FOR HEMOPHILUS PERTUSSIS FOLLOWING WHOOPING COUGH AND FOLLOWING IMMUNIZATION. *Paul F. de Gara,* Cornell University Medical College, New York.

The agglutinative reaction for *Hemophilus pertussis* was found to be positive in 34.2 per cent of children who had a history of pertussis. The longest interval between recovery from the disease and testing with positive result was 8 years.

Following prophylaxis with bacterial vaccines, agglutinins were found in 93.5 per cent. Some of the determinations were made as long as 9 years after immunization. Prophylactic injections of "pertussis antigen" were rarely followed by high agglutinin titers. Stimulating injections of pertussis vaccines evoked a notable rise of circulating agglutinins in 97.4 per cent.

There was some correlation between the results obtained by slide agglutination and test tube agglutination, but the slide test alone did not always differentiate between low and high agglutinin titers.

Of 17 untreated control patients, 16 had no agglutinins and 1 had agglutinins in low titer.

OBSERVATIONS CONCERNING THE GROWTH AND METABOLIC ACTIVITIES OF MYXOCOCCI IN A SIMPLE PROTEIN-FREE LIQUID MEDIUM

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Although a great deal seems to be known concerning the mode of growth of myxococci upon solid media (for a summary of existing knowledge see, for example, Knaysi, 1944) there is surprisingly little information in the literature about their propagation and metabolic activities in homogeneous, chemically defined liquid media. Attention has recently been paid to their ability to grow upon and lyse eubacteria in suspensions of known electrolyte content (Singh, 1946). The lysis of dead eubacteria under these conditions seems to be chiefly due to exocellular proteolytic enzymes elaborated by the swarming myxococci, but Singh (1946) has proved that fully viable eubacteria are in certain instances also lysed. It is therefore possible that myxococci may sometimes produce true nonenzymic antibiotic substances, capable of killing viable eubacteria and thereby rendering them susceptible to lysis. In a search for these hypothetical antibiotic substances some typical myxococci have now been grown in a homogeneous protein- and polysaccharide-free medium of simple composition in which amino acids provided the sole source of both carbon and nitrogen. As anticipated, the occurrence of at least one antibiotic substance of considerable activity has been demonstrated, and its separation in the monamino-monocarboxylic acid fraction is described. Some observations, which appear to be new, concerning the peculiar mode of growth of myxococci in homogeneous liquid media are also recorded.

EXPERIMENTAL

1. *Cultures and method of assay for antibacterial potency.* Among a dozen cultures of myxococci and related chondrococci obtained from the Department of Soil Microbiology, Rothamsted Experimental Station, four of the former and one of the latter could be grown in the simple amino acid medium described in section 4. Only two of these, however, were of sufficient biochemical interest to warrant further investigation at the moment—namely, two distinct strains of *Myxococcus virescens* (Bergey *et al.*, 1939), one of which, strain A, produced an antibiotic substance, and the other, strain G, secreted an active gelatinase but no antibiotic substance.

Antibacterial activity was measured by the serial dilution method using *Staphylococcus aureus* (Heatley strain; N.C.T.C. no. 6571) as the test organism. The usual series comprised the following dilutions: 5, 14, 40, 70, 85, and 125, and was made up by adding 1.0, 0.3, and 0.1 ml of clear metabolic solution, respectively, to 4-ml quantities of heart broth containing no added sugar, and then transferring 1.0, 0.8, and 0.5 ml, respectively, from the second tube to further 4-ml quantities of heart broth. One loopful (0.004 ml) of a well-shaken and 1,000-fold-diluted 20-hour culture of *S. aureus* in heart broth was used to inoculate each tube, and readings were made after 1 and 2 days' incubation at 37 C.

2. *Measurement of gelatinase activity.* Accurate determination of the absolute gelatin-liquefying power was not deemed necessary in this investigation (see Haines, 1932, for a discussion of the difficulties involved in such determinations). All that was required was a quick method for assigning the correct relative gelatinase activities to the various members of a series of preparations put up at the same time. The following method, which required no special media or apparatus, was found to be adequate: Ordinary sterile 12 per cent nutrient gelatin medium, as used for bacterial stab cultures (8-ml lots in 6-inch test tubes) was first completely liquefied by slowly heating to 45 C in a water bath. The flame was then removed and cold water added to the bath to reduce the temperature to 40 C. When the temperature had further fallen to 37 C by ordinary cooling, 1.0 ml of the clear metabolic liquid under test was added to 8 ml gelatin and the tube well shaken. Three drops of chloroform were then added, and the whole was well shaken again to saturate the gelatin with chloroform. The series of tubes so prepared (each specimen being tested in duplicate) was immediately transferred to the cold room for 15 minutes for the gelatin to set, then it was incubated at 24 C, and readings were taken after 6 and 24 hours. Good liquefaction required 6 hours or less; moderate liquefaction required overnight incubation. Control tubes containing 1.1 ml of uninoculated medium invariably remained firm even after several days' incubation.

3. *The maintenance of fruiting cultures of myxococci on a solid medium of simple composition.* It is well known that myxococci do not in general form fruiting bodies on ordinary nutrient agar, and that the vegetative bacterial growth on this medium, being liable to autolysis within 10 to 15 days and being also tenaciously adherent to the agar before autolysis, is not very suitable for the inoculation of liquid media. Contrary to expectations aroused by the literature, the species here studied did not need special decoctions of dung or of eubacterial cells in order that a supply of long-lived cultures, containing both fruiting bodies and swarming rods, might be maintained. The organisms gave scanty but typical growth, with numerous fruiting bodies, on slopes of sucrose Czapek agar of the following composition: sucrose, 0.5 per cent; $NaNO_3$, 0.2 per cent; K_2HPO_4, 0.1 per cent; KCl, 0.05 per cent; $MgSO_4 \cdot 7H_2O$, 0.05 per cent; $FeSO_4 \cdot 7H_2O$, 0.001 per cent; and agar, 2 per cent. When an inoculation was made at the top of such a slope, swarming invariably took place at 24 C—most evenly when the surface of the agar was dry—with abundant production of tiny fruiting bodies, usually well behind the advancing edge of growth, which could easily be detached from the medium, either by striking gently with a loop or by shaking with a little sterile water. The bacterial growth was very thin indeed and traveled at so slow a rate that weeks elapsed before a 2-inch-long slope was covered. The practice has been to use almost the whole of the growth on a 3- or 4-week-old slope at one

time, in order to inoculate two or three tubes of liquid medium, each receiving the growth from an equal area on the slope. What was left of the growth was finally transferred to another sucrose Czapek agar slope. In this way an adequate supply of viable growth for the inoculation of liquid media at any time could be maintained. For larger volumes of liquid medium (100 ml or more) the whole growth from a slope was used as inoculum, after emulsification in a little sterile water.

4. *Composition of the simplest liquid medium which will support growth of myxococci.* Attempts to grow myxococci in liquid media with a sugar as the source of carbon, together with inorganic nitrogen, failed completely. Even with the amino acid medium described below, the addition of glucose or sucrose, sterilized separately, did not result in any increase of growth or production of characteristic metabolic products, such as an antibiotic substance or gelatinase. The sugars appeared to remain unfermented, even when good growth took place.

After many trials a basal medium was hit upon which would allow a just perceptible development of a film of growth of *M. virescens* A at 24 C, with a correspondingly small but detectable production of antibacterial activity in the metabolic solution, i.e., when 1.0 ml of the clear supernatant after a week's incubation was added to 4 ml of heart broth. This basal medium had the following composition: asparagine, 0.44 per cent; total acid hydrolyzate of casein free from polypeptides and carbohydrates (in powder form as supplied by Glaxo Laboratories, Ltd.), 0.06 per cent; K_2HPO_4, 0.4 per cent; NaCl, 0.2 per cent; and $MgSO_4 \cdot 7H_2O$, 0.02 per cent. It had a pH of 7.8, and had not the slightest inhibitory effect upon the growth of *Staphylococcus aureus* when it was added to heart broth to the extent of 30 per cent. In exploratory experiments 5-ml quantities of this filtered medium in 6-inch test tubes were sterilized by steaming, and after inoculation the tubes were well shaken and incubated in a sloping position for the reason mentioned in section 5b. To render the growth more evident, after decantation of the supernatant and washing once with distilled water, it was stained with filtered 0.1 per cent methylene blue for 10 minutes, again washed, and the tube allowed to dry at room temperature in an inverted position. The extent and intensity of the blue stain in the lower part of the tube was a measure of the filmlike growth of the myxococcus, all of which usually adhered to the glass. Although microcysts were formed in abundance in 14 days, the production of macroscopic fruiting bodies was observed under these conditions with *M. virescens* G only. The growth on submerged glass may therefore sometimes be different in appearance from that on agar.

Myxcocci may be maintained in serial cultivation in this liquid medium provided the sowing is made from a culture at least 2 weeks old and that the growth is first thoroughly broken up by shaking in order to obtain a suspension of free microcysts. When in doubt, a stained preparation should be made from a loopful of the well-shaken culture.

There was no evidence that the asparagine in the foregoing basal medium served as a nutrient or was fermented. The change in pH and the production of ammonia was trifling even when the growth was good (see below). As far as could be judged, all metabolic solutions gave as intense a bluish-purple color with copper sulfate and caustic soda under the conditions of the biuret test as did the unsown medium. This color which is quite different from that given by polypeptides, was found to be due to asparagine and to no other constituent of the medium. The asparagine may therefore be regarded as an inert inorganic buffer, tolerated by myxococci, the casein hydrolyzate being the real nutrient. It is not yet known which amino acids are really essential for growth, since no artificial mixture of pure amino acids has so far proved adequate. Experiments along these lines are being continued.

Attempts to improve the medium so that it would support better growth of, and development of antibacterial activity with, *M. virescens* A succeeded only when casein hydrolyzate was substituted for all or part of the asparagine. For example, other things being equal, the film of growth was obviously many times thicker when the concentration of casein hydrolyzate was 0.85 per cent than when it was 0.06 per cent as in the basal medium. Not all pure amino acids are tolerated when added to the basal medium at a concentration of 0.4 per cent. Sodium glutamate was as readily tolerated as asparagine (glutamic acid is in fact the chief constituent of hydrolyzed casein). The basic amino acids arginine, histidine, and lysine were also tolerated, but glycine, alanine, or cysteine hydrochloride, separately or admixed, entirely prevented growth of *M. virescens* A when present in the medium at a concentration of 0.4 per cent. Finally, the addition of 5 mg of a vitamin mixture of the following composition—nicotinic acid, 1.5; riboflavin and pyridoxine, 1 each; calcium pantothenate and *p*-aminobenzoic acid, 0.5 each; aneurin, 0.4; and biotin, 0.00025 mg—to 100 ml of basal medium had no perceptible effect in stimulating growth or production of antibiotic substance.

5. *Physical factors influencing the growth of myxococci in liquid media.* When growth occurs from an inoculum of microcysts added to a clear, liquid, amino acid medium free from solid particles (see last section), there is, of course, no development of a turbidity, emulsifiable deposit, or surface pellicle as with eubacteria, but rather of a widespread and surprisingly coherent submerged film of bacteria. This film, the thickness of which depends on the concentration of nutrients in the medium, adheres rather tightly to the whole of, or to only a part of, the wet glass surface of the containing vessel, depending on its shape. Certain purely physical factors, which need not be considered in connection with the growth of ordinary eubacteria in liquid media, have therefore to be reckoned with. Some of these factors are:

(a) *Depth of liquid.* This is important, not so much because it affects the oxygen supply to the lower layers of liquid (for growth will occur quite readily at the bottom of a 6-inch test tube nearly filled with medium), but because it determines the speed at which the added microcysts will make stable contact with the submerged glass surface and thereafter serve as foci of growth. The following experiment showed that the microcysts tended to settle slowly: Several 6-inch test tubes half-filled with sterile medium were inoculated from slopes as described in section 3, and an even suspension of microcysts was produced in each by vigorous shaking. They were incubated in the upright position for a day, then a number of them were inclined almost to the horizontal position, the rest being undisturbed, and the incubation was continued for 10 days. Only those tubes which had been inclined showed growth on the submerged wall of the tube as well as on the bottom. The upright tubes were then inclined and further incubated, but no great spread of growth took place, presumably because there were no more viable microcysts in suspension to settle on the extra, almost horizontal glass surface thus provided. It may be concluded that, if only a small inoculum is to be given, the layer of liquid medium should be as shallow as possible.

(b) *Availability of the submerged glass surface and the importance of the ratio of available surface to volume of medium.* Although myxococci can swarm vertically, i.e., against gravity, up an agar slope, none of the strains here studied could do so to any appreciable extent up a vertical, or steeply sloping, submerged glass surface. Two reasons for this may be advanced: (1) Few of the microcysts from the inoculum can stably settle on such inclined surfaces to provide foci of growth (see above). (2) Good growth from a given focus, even on a horizontal submerged glass surface, seems never to extend for more than a few mm in any direction. Hence the desirability of a massive inoculation with microcysts evenly distributed by shaking.

It follows that if the medium is contained in an ordinary flat-bottomed Erlenmeyer flask or in a cylindrical vessel such as the Glaxo container formerly used for penicillin production (Clayton *et al.*, 1944), the only part of the submerged glass available for supporting growth is the floor of the vessel. With such a cylindrical container 17 cm in diameter, containing liquid to a depth of 1 cm (a shallower layer than this is scarcely desirable), the horizontal floor area available for supporting growth of the myxococcus is 230 sq cm, the submerged vertical walls not so available occupy 54 sq cm, and the available surface per unit volume (hereafter referred to as A_v and expressed as sq cm per ml of liquid medium) is 1 sq cm, or $A_v = 1$. With a vessel of this type there is no practicable means of increasing A_v, and any increase in the volume of the medium will lower it. A_v becomes very small indeed in the case of an upright test tube half-filled with medium, but approaches unity if the test tube is held almost horizontally, when the greater part of the submerged glass becomes available to the myxococcus. The following device has been employed to increase A_v to 1.5 with a vessel holding 100 ml of medium: A liter, round-bottomed, spherical bolthead flask 13 cm in diameter contained liquid to a depth of 2.5 cm, giving roughly 100 sq cm of submerged glass, all of which is available to the organism (as proved by experiment) since the upward slope is never great. Extra available glass surface was supplied by the introduction of three 7.6-by-2.5-cm microscope slides, arranged almost horizontally below the surface of the liquid, two whole slides being side by side and the third, broken into two pieces, inserted below them. Naturally only the upper surface of the slides is available to the myxococcus, so that the extra surface available is $3 \times 7.6 \times 2.5 = 57$ sq cm. In using this arrangement it is best to sterilize the flask containing medium with the slides inside, inoculate, shake well, and then quickly arrange the slides in the desired position by means of a sterile glass rod. One further advantage is that slides may be withdrawn aseptically from time to time and the growth upon them, which adheres to the glass so tenaciously that fixation is not required, examined microscopically after washing and staining with methylene blue, as described in section 4. The film of growth is usually rather too thick for profitable examination with the higher magnifications, but this difficulty might be overcome by cutting down the casein hydrolyzate in the medium to the absolute minimum.

It should be added that one strain, *M. virescens* G, did sometimes form very small elements of surface growth, apparently supported by surface tension, and occasionally also patches of growth spreading downwards from the surface over the submerged vertical glass wall of a Glaxo container. The total area of such additional growth was, however, quite small in comparison with the growth on the floor of the vessel. As shown in the next section, the value of A_v ought to be as large as possible, not only for the maximum amount of growth but also to ensure the maximum metabolic activity.

TABLE 1

Antibacterial activity of metabolic solutions of M. virescens A at various stages of incubation

PERIOD OF INCUBATION	LIMITING DILUTION OF CLEAR METABOLIC SOLUTION IN HEART BROTH COMPLETELY INHIBITING S. AUREUS AT 37 C FOR:	
	1 day	2 days
days		
0	No effect	
2	No effect	
3	5	No effect
5	14, 40	14, 14
6	40, 40	14, 14
7	40	14
8	14, 40	14, 14
10	14, 40	14, 14
13	14	5
19	5	No effect
23	5, no effect	No effect
27	No effect	No effect

The medium contained casein hydrolyzate, 0.1%, and asparagine, 0.4%, and was distributed in 50-ml lots in a large number of 250-ml Erlenmeyer flasks, which were incubated side by side at 24 C.

6. *Production of antibiotic substance by M. virescens A.* The following facts have been established concerning this substance (or substances):

(a) The data summarized in table 1 show that it is not a stable product of metabolism, since it tends to disappear if the incubation is unduly prolonged—beyond 10 days.

(b) The better the growth, i.e., the greater the concentration of casein hydrolyzate in the medium, the better the production of antibiotic substance (table 2).

(c) For a given concentration of casein hydrolyzate in the medium, the greater the value of A_v (area of available submerged glass surface per unit volume of medium) the greater the concentration of antibiotic substance formed (table 3).

(d) The antibacterial effect is not due to an enzyme since solid products of considerable activity have been obtained which are soluble in ethyl or butyl alcohol (see next section) and which have no proteolytic action at all. This was shown very simply by adding Esbach's protein reagent to those *S. aureus* assay tubes which remained clear after a week's incubation at 37 C. Those to which bactericidal metabolism solution, containing also protease, had been added gave no precipitate, but those to which a solution of the alcohol-soluble product had been added gave heavy precipitates, just as with sterile uninoculated heart broth itself.

TABLE 2

Effect of casein hydrolyzate on antibacterial activity of metabolic solutions of M. virescens A

CONCENTRATION OF CASEIN HYDROLYZATE	ANTIBACTERIAL TITER (AS IN TABLE 1)	
	1 day	2 days
%		
0.06	5	5
0.1	14	14
0.33	70	40
0.5	200	85
0.85	200	70

The experiments were conducted with 5-ml quantities of medium in sloping 6-inch test tubes which were incubated for 11 days at 24 C. When the initial concentration of casein hydrolyzate was below 0.5%, asparagine was added to the medium to bring the total concentration of amino acids up to 0.5%.

TABLE 3

Effect of volume of liquid medium in a Glaxo container 17 cm in diameter upon production of antibiotic substance from 0.1 per cent casein hydrolyzate

VOLUME OF MEDIUM	A_v (AVAILABLE SURFACE/VOLUME)	ANTIBACTERIAL TITER (AS IN TABLE 1)	
		1 day	2 days
ml			
200	1.1	40	14
300	0.75	14	5
400	0.6	14	5
600	0.4	5	No effect
900	0.25	No effect	

The incubation period was 11 days in each instance. The floor of the vessel was covered with growth always, but not the vertical walls.

(e) Seitz filtration removed part but not all of the antibacterial substance from an active metabolic solution. This substance was far more stable to heat at 60 C than the gelatinase also present in the metabolism solution (see section 8).

7. *Preparation of a crude solid product containing the antibacterial activity.* The antibiotic substance rapidly disappeared from the separated metabolism solution unless the latter was stored in the cold room. It appeared to be insoluble in the usual nonhydroxylic organic solvents at all pH values (or else inactivation took place during the extraction process), nor could it be eluted from charcoal without inactivation. The only feasible mode of extraction so far discovered was to evaporate the filtered metabolic solution to small volume *in vacuo* below 45 C, then immediately to take it down to dryness in a vacuum desiccator over conc. H_2SO_4, and to extract the solid so obtained with 100 parts of absolute ethanol at 55 to 60 C for an hour, with stirring from time to time to reduce the original gummy material to a state of fine division. The whole was then filtered and the filtrate evaporated to dryness in a vacuum desiccator at room temperature. In a typical experiment the contents of a Glaxo container, which originally contained 300 ml of medium (casein hydrolyzate, 0.33 per cent; asparagine, 0.3 per cent), were filtered after 12 days' incubation when the 1-day *S. aureus* titer was 70. The solid product obtained by evaporation of the metabolic solution weighed 2.8 g, and a 1 per cent aqueous solution of a small portion of it, after heating at 65 C for 6 minutes to kill extraneous eubacteria, had roughly the expected antibacterial activity (1-day titer = 40). An alcohol extraction, conducted on 200 mg as described above, yielded 13 mg of colorless solid, which gave the ninhydrin and other reactions for amino acids. One part of this solid in 12,500 parts of heart broth completely inhibited the growth of *S. aureus* for 2 days under the ordinary assay conditions. But little activity remained in the alcohol-insoluble residue, so considerable inactivation had taken place during the alcohol extraction. The active product, like the original metabolic solution, had no detectable inhibitory action on gram-negative bacteria.

The optimum temperature for the alcohol extraction seems to be 55 to 60 C. When boiling alcohol was used, the yield was greater but the product was, weight for weight, considerably less active, whereas an extraction at 37 C gave a smaller yield of a product with about the same activity. The further purification of the antibiotic substance is in progress. No advantage accrues by the use of dry butanol in place of ethanol, and wet butanol causes complete inactivation.

8. *Gelatinase production by myxococci in a protein-free medium.* Like certain eubacteria, species of *Myxococcus*, particularly *M. fulvus* and *M. virescens*, secrete gelatinase in a protein-free liquid medium, in this instance the amino acid medium previously described. The enzyme, as produced by the strains studied in this investigation, was very heat-labile, and at pH 7 to 8 was destroyed by heating the metabolic solution for 10 minutes at 60 C. Metabolic solutions of *M. virescens* G which were active in liquefying gelatine in 6 hours at 24 C under the conditions described in section 2 were also capable of clearing suspensions of killed gram-negative eubacteria (rendered nonviable by heat, by chemical poisons such as chloroform, or by age in the ordinary way) of con-

siderable opacity; e.g., an almost water-clear liquid resulted from the incubation for 6 hours at 37 C of a mixture of equal parts of the metabolic solution and a distilled water suspension of *Escherichia coli*, killed at 100 C, of opacity of 400 to 800 *m* according to Peskett's (1927) $BaSO_4$ standards. There was, however, no visible effect during the same length of time upon a suspension of viable coliform bacteria, freshly prepared from a young nutrient agar slope. The lytic enzyme was truly exocellular since it was found in a Seitz filtrate of a strongly proteolytic metabolic solution.

There remains the further question whether myxococci, like eubacteria, secrete gelatinase only in a medium containing calcium ions (Haines, 1931, 1932, 1933). The casein hydrolyzate used in this research was not quite calcium-free, nor has any simple method been yet devised for removing the calcium from it without impairment of its nutrient value toward myxococci. The latter have, however, been grown in washed and heat-killed suspensions of *Pseudomonas fluorescens* in distilled water, which were presumed to be calcium-free because the organism had been maintained for many subcultures in a liquid synthetic glucose nitrate Czapek-Dox medium (at pH 7) containing no calcium, since all the inorganic salts used were the purest obtainable. The metabolic solutions so prepared undoubtedly contained gelatinase, and there was no increased production of the enzyme observed when calcium chloride was added to the distilled water suspension of the washed pseudomonads before inoculation with microcysts of *M. virescens*. No case could therefore be made for any important role played by the calcium ion in the production of exocellular myxococcal proteinase.

DISCUSSION

A consideration of the solubilities of amino acids in absolute alcohol (Cohn and Edsall, 1943) shows that the antibiotic substance must have been extracted by this solvent together with certain monamino-monocarboxylic acids, chiefly valine and the leucines, which together constitute nearly 20 per cent of the total amino acids of hydrolyzed casein. At 25 C, for example, the volume of alcohol used in the extraction (20 ml) would dissolve about 2 to 3 mg each of valine and the leucines, and no doubt the solubilities of these amino acids are not less than this at 55 C. It is highly probable, therefore, that the greater part of the 13 mg of active solid obtained by alcohol extraction was in fact made up of valine and the leucines; hence the activity of the antibiotic substance, when obtained in the pure state, ought to be many times greater than the best value recorded in this paper.

Although the limited survey of the antibacterial activities of myxococci here described has yielded disappointing results, in that only one antibiotic substance has come to light, yet it might be well worth while to extend the study to include other genera in the great group of myxobacteria. The soluble proteolytic and bacteriolytic enzyme elaborated by myxococci also deserves further study since it may not be identical with the corresponding eubacterial enzyme. In particular, the inorganic ions required for its formation should be determined since they may be different from those required for the production of eubacterial proteinase.

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SUMMARY

Two strains of *Myxococcus virescens* will grow in a simple liquid medium containing the amino acids of a total acid hydrolyzate of casein as the sole source of carbon and nitrogen. They then form a coherent film in contact with all or part of the submerged glass surface, depending on the shape of the vessel, but are not able to spread vertically to any appreciable extent on wet glass. Gelatinase is secreted under these conditions, and in one instance a nonenzymic, alcohol-soluble, chemically labile antibiotic substance was produced also, active against gram-positive eubacteria only. Further quantities of asparagine, sodium glutamate, or the basic amino acids are tolerated by this myxococcus, when they are added as supplements to the casein hydrolyzate medium, but other amino acids such as glycine, alanine, or cysteine, if present in sufficient concentration, are definitely inhibitory to its growth. The antibiotic substance is associated with the valine and leucine components of the monamino-monocarboxylic acid fraction.

The production of gelatinase by myxococci probably does not need the presence of calcium in the medium as with eubacteria. Myxococcal metabolic solutions which contain gelatinase, but no antibiotic substance, are also markedly bacteriolytic toward nonviable gram-negative bacteria, but have no corresponding action on viable bacteria.

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THE EGG YOLK PLATE REACTION FOR THE PRESUMPTIVE DIAGNOSIS OF CLOSTRIDIUM SPOROGENES AND CERTAIN SPECIES OF THE GANGRENE AND BOTULINUM GROUPS

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The original Nagler (1939) reaction consisted of mixing dilutions of the toxin of *Clostridium perfringens* with human serum and noting the appearance of an opalescence. MacFarlane, Oakley, and Anderson (1941) reported that crude lecithovitellin from egg yolk gave a stronger reaction than serum, and postulated that the reaction was due to the alpha toxin of *C. perfringens*. Crook (1942), van Heyningen (1941), and others have used the tube reaction extensively. The test is now referred to as the Nagler or LV reaction.

In a study of the use of the tube reaction for rapid identification of *C. perfringens*, Hayward (1941) mentioned that cultures of *C. perfringens* on agar containing human serum produced a well-defined opacity extending from the edge of the colony. The reaction was completely inhibited by the addition of homologous antitoxin to the medium. Strains of *Clostridium oedematiens* and the *Clostridium bifermentans–Clostridium sordelli* group also produced similar zones, whereas other species were negative. The reactions of the *C. bifermentans–C. sordelli* group were inhibited by *C. perfringens* antitoxin. In 1943 Hayward presented more extensive studies with the plate reaction and answered the criticisms of Weed and Minton (1943) concerning the nonspecificity of the tube reaction. Hayward concluded that human serum was easier to obtain and was preferable to egg yolk in the plate reaction. It was suggested that 4 per cent concentrations of Evans, Lescher, and Webb peptone could be substituted for the Fildes broth in the medium.

Nagler (1944, 1945) has described a reaction, similar in some respects to the above, for the recognition of *C. oedematiens* (*C. novyi*). The medium used was a peptic digest of ox muscle to which egg yolk and defibrated sheep blood were added. On this medium surface colonies of *C. oedematiens* are surrounded by two distinct zones. The first opaque hemolytic area is surrounded by a dark red zone, referred to as a zone of reduction. A mother-of-pearl luster film covers both the colonies and the zones. In contrast to the foregoing reaction given by type A cultures, a dark red zone without luster was produced by one strain of type B (differentiated, according to Scott, Turner, and Vawter, 1934, and Turner and Eales, 1943, from type A, not on toxin specificity but on cell size and glycerol fermentation) and two of type C (Kraneveld's bacillus of osteomyelitis bacillosa bubalorum) of *C. oedematiens*, and a strain of *Clostridium hemolyticum*. A pearly film, but no dark red zone, was produced by *Clostridium sporogenes*, *Clostridium parasporogenes* and *Clostridium botulinum* (probably *C. parabotulinum* in the Bengston, 1924, terminology). The reaction characteristic of *C. oedematiens* was not inhibited by homologous antitoxin.

EXPERIMENTAL

In connection with other studies, it has been our purpose to consider these plate reactions as a means of presumptive recognition of certain clostridia, using adequate numbers of authentic strains of certain species to study the specificity of the lecithinase reactions. We have used human serum at times and confirmed the statements concerning the reactions with it. In plate tests, however, the more intense reactions obtained with the egg yolk appear to be of advantage. With serum the zones of precipitation are considerably less dense, and the luster areas, to be described, are less easily recognized.

Preparation of egg yolk suspension. Scrub and sterilize (dilute $HgCl_2$) the shell of a fresh hen's egg. Aseptically withdraw, by aspiration, the yolk (after the white has first been removed) to a sterile tube which is then closed with a rubber stopper. Add an equal volume of sterile saline and mix by inverting the tube several times. If preserved with merthiolate (1:10,000), the preparation will be usable for 10 to 14 days. Before use, test sterility by plating 1 ml in nutrient agar.

Medium. In substitution for the digest media suggested by Hayward (1941, 1943) and Nagler (1945) the following medium, prepared from commonly available ingredients, is recommended: proteose peptone no. 2, 40 g; Na_2HPO_4, 5 g; KH_2PO_4, 1 g; NaCl, 2 g; $MgSO_4$, 0.1 g; glucose, 2 g; agar, 25 g; and distilled water, 1,000 ml. Adjust pH to 7.6 and sterilize for 20 minutes at 240 F. Add 10 ml of yolk suspension to each 90 ml of medium and, for best results, pour 15 ml in 100-mm diameter plates. Streak plates in such a manner that well-isolated colonies will be obtained. Incubate plates, in anaerobic system of preference, for 48 to 72 hours. Positive results, particularly with *C. perfringens*, may appear earlier.

Other agar media tested included beef heart infusion, liver infusion, casamino acids, N-Z case, trypticase, proteose peptone, proteose peptone no. 3, bacto peptone, thio-peptone, nutri-peptone, yeast extract, sodium caseinate, amigen, and peptic digest of beef liver and beef muscle. The peptones and similar products were tested, with the salts listed above, etc., in 1, 2, and 4 per cent concentrations. In all instances the 4 per cent concentration was superior to the other two. Without giving the results in detail, we may mention that the heart infusion, the peptic digests, and certain peptones (trypticase, bacto peptone, proteose peptone, and proteose peptone no. 3) gave sufficiently good results to indicate that they might be used with the plate reaction. In general, it appears that any medium which supports satisfactory growth and provides suitable conditions for toxin production probably could be used.

Species Identification Reactions

Clostridium perfringens (*C. welchii*). The colonies are round and smooth (rough variants excepted) and are surrounded by a wide (8-mm) zone of opaque white precipitate (figure 1, numbers 1, 2, 3, and 4). There is no luster, and after the colonies have stood at room temperature in the air for several hours, extra zones appear at the edge of the previous zone with the final outer edge more

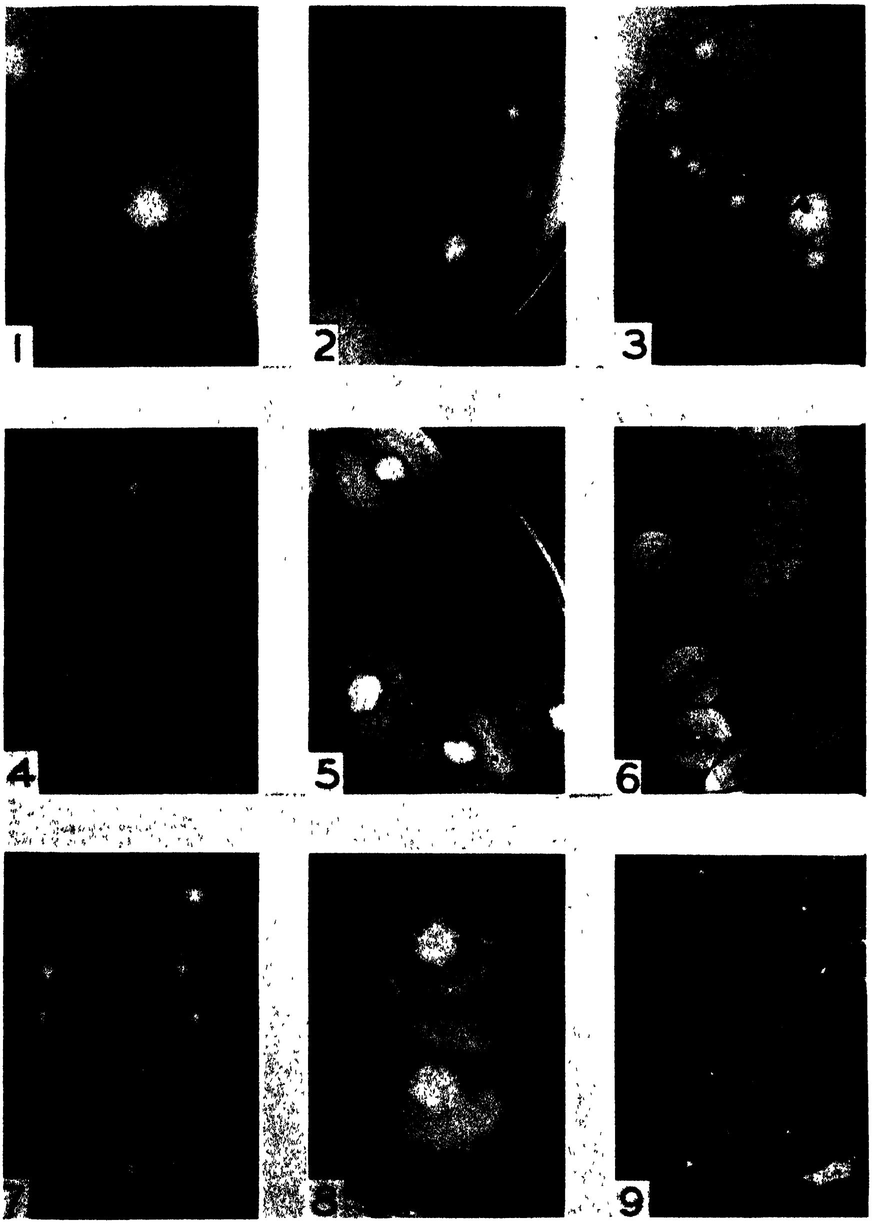

Fig. 1. Colonies of Various Clostridia on Egg Yolk Agar

Magnification in all cases is approximately 3 X. The luster areas described in the text do not show. No. 1, *Clostridium perfringens* type A. No 2, *Clostridium perfringens* type B. No. 3, *Clostridium perfringens* type C. No. 4, *Clostridium perfringens* type D. This illustrates the smaller zone of precipitation sometimes encountered with this species. No. 5, *Clostridium novyi* type A. No. 6, *Clostridium novyi* type B. No. 7, *Clostridium tertium*. No. 8, *Clostridium sordelli*. No. 9, *Clostridium tetani*.

intense than the remainder of the area, but the line of demarcation is not a sharp one. The reaction is given by all four toxin types (Oakley, 1943). Each of 50 strains which we have tested give the typical picture, although there is some variation in the size of the zone of precipitation.

Clostridium novyi (*C. oedematiens*) type A. The reactions were given by each of 61 strains classed as *C. novyi* by other reactions. Colonies are smooth, with irregular edges, and show a precipitation zone under the colony and in a regular circle to a radial distance of 4 mm (figure 1, number 5). The precipitation is more intense than with *C. perfringens*, and the edge of the zone is sharply defined. The characteristic feature is an irridescent luster area, marked by radial linear striations, covering the colony and extending beyond over the surface of the agar in a regular circular zone to a radial distance of about 2 mm, only partially covering the precipitation zone. After a further period, an additional zone of intense luster appears, which is banded by a less intense area. Additional concentric zones of precipitation may appear around the original. In three cultures a slightly different type of reaction was observed immediately after the plates were removed from the anaerobic environment. With these, the colony is more or less rough and larger, and the luster zone is narrow and follows the contour of the colony. The radial striations are less marked. The precipitation extends beyond the luster in a regular circle. After an additional period, the reaction is similar to that described for the others.

Clostridium novyi (*C. oedematiens*) type B. Small irregular, transparent colonies produce a wide (8-mm) regular circle of precipitation under and beyond the colony. The edge of the zone is sharply defined (figure 1, number 6). No luster is evident immediately or later. After the plates have been exposed for several hours, a larger zone of precipitation is present, with the original zone outlined by a heavy ring of precipitation. Eight strains were tested, and all gave the reaction described.

The Clostridium sordelli Clostridium bifermentans group. Small-to-medium-sized colonies, which are slightly raised and shiny and have rough edges, produce no luster but a wide zone of precipitation (figure 1, number 8). The reaction is practically indistinguishable from that of *C. perfringens*. Later, one or more additional zones of precipitation may appear beyond the original zone. These reactions were given by 36 strains. It is of importance that the same reaction is given by the nontoxic *C. bifermentans* type as by the toxic *C. sordelli* type. This and other evidence obtained in collaboration with Helen Michael indicates that the production of a precipitate from egg yolk does not parallel the production of the lethal toxin by *C. sordelli*.

Clostridium hæmolyticum. This species gives a punctiform colony with a wide area of intense precipitation surrounding the colony.

Clostridium sporogenes. The precipitate is deposited under the colony and rarely spreads beyond (figure 2, number 12). A slight luster covers the colony but does not extend beyond. In the usual type of colony the rhizoids may extend beyond the luster and precipitate. After an additional period, the edge of the precipitate is marked by a zone of more dense precipitation. In some cases there is a slight clearing of the medium in a narrow band beyond the colony edges; this may be surrounded by a zone of faint precipitation. This reaction has been given by approximately 100 strains. A slight variation from this has been noted with approximately 50 strains for which a tentative designation

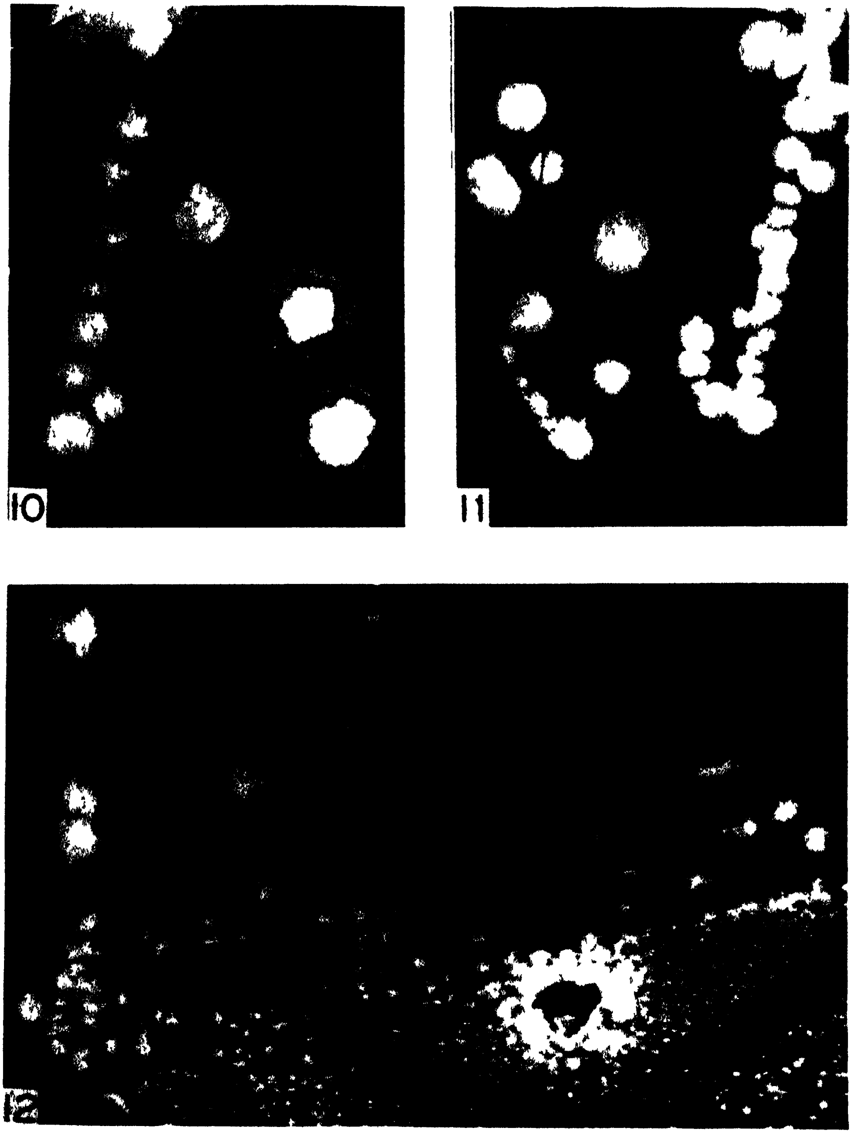

FIG 2

No 10, *Clostridium botulinum* types C, D, or E No 11, *Clostridium parabotulinum* type A or B. No 12, *Clostridium sporogenes*.

to this species has been made on other grounds. In these, the colony is more regular, and the luster and the precipitate do not extend beyond the colony. In three cultures, which may, however, not be *C. sporogenes*, we have observed no reaction.

Clostridium parabotulinum types A and B (Bengston, 1924, terminology). The raised, irregular-edged colonies are covered with a luster which extends in a regular circle slightly beyond the colony edge. An area of precipitation lies under the colony and to the edge of the luster zone (figure 2, number 11). A slight clearing of the medium beyond the precipitation may be noted. Although the radial striations are present, they are not so marked as in the *C. novyi* reaction. At a later period, there may be a slight precipitate beyond the luster zone, but this is indistinct. The reaction is given by nontoxic strains, identifiable by physiological and agglutination reactions, as well as by the toxic cultures.

Clostridium botulinum type B. The colonies are flat and spreading with irregular edges. The reaction is essentially the same as for *C. parabotulinum* although the reaction zones tend to be wider. Three strains have been studied.

Clostridium botulinum types C, D, and E. In these cultures the flat, irregular-edged colonies are surrounded by a wide zone of precipitation as shown in figure 2, number 10. There is also a narrow luster zone which follows the contour of the colony edge. The regular circle of precipitation extends well beyond the luster. The edge of the precipitation is not so sharply defined as with *C. novyi*. Later, an additional luster zone appears and somewhat indistinct zones of precipitation may be formed. These reactions were given by two strains of type C and one strain each of types D and E.

Other species. Strains of the following species of *Clostridium* have failed to show a luster, a zone or precipitation, or any other identifying reaction on the medium other than the usual colonial morphology: *C. tetani* (figure 1, number 9), *C. septicum* and *C. tertium* (figure 1, number 7), *C. histolyticum*, *C. capitovalis*, *C. chauvoei*, *C. cochlearium*, *C. butyricum* (physiologically closely allied to *C. perfringens*), and *C. acetobutylicum*. For the latter two organisms the glucose concentration in the medium was increased to 1 per cent. Crook (1942), using the tube reaction, obtained negative results with *C. septicum*, *C. histolyticum*, and *C. tetani*, and also with one strain of *C. botulinum*. With the latter species (*C. parabotulinum* of the American literature) we have obtained positive reactions with the plate technique but negative results with the tube method. Also, although Crook reported positive results with *C. chauvoei*, we have found this organism to be negative in the plate test.

We have obtained positive results with various aerobic organisms, particularly *Actinomyces*, *Aspergillus*, and certain members of the genus *Bacillus*. In the *Bacillus* group positive results (precipitation but no luster) were obtained with strains designated *B. lacticola*, *B. tumifaciens*, *B. ellenbachensis*, *B. megatherium*, *B. cereus*, *B. mycoides*, and several unidentified cultures which appeared as contaminants, but negative results with *B. graveolens*, *B. subtilis*, *B. circulans*, *B. brevis*, *B. anthracis*, *B. ruminatus*, *B. rotans*, *B. alvei*, *B. globigii*, *B. aterrimus*, and *B. silvaticus*.

Inhibition of Reactions by Antiserum

It appears that no statements concerning this question will be of value until more information is available based upon the use of sera of known antilecithinase content. Commercially prepared antitoxin should not be used, without assay, to inhibit the reaction of a newly isolated strain in the divided plate technique in which one-half of the plate is spread with antiserum to inhibit the characteristic reaction (Hayward, 1943, 1945; War Wounds Committee, 1943). The antilecithinase properties of a serum may not bear a positive correlation to the antilethal value, and it is presumed that the latter value has been the one considered in the standardization of antitoxic sera. Certain samples of commercial sera seem to be almost completely lacking in antilecithinase properties. This may explain the failure of antitoxin inhibition of the *C. oedematiens* reaction on blood-egg-yolk agar reported by Nagler (1944, 1945). Also, as Hayward (1943) has pointed out, the antitoxin of *C. perfringens* inhibits the reaction of the *C. sordelli–C. bifermentans* group. Our results confirm this and reveal evidence of other examples of this phenomenon.

Specificity of the Reaction

The LV reaction was originally thought to be specific for *C. perfringens*, though this concept has been dispelled by the results of Hayward (1941, 1943) and Crook (1942), and by the foregoing results. One may logically question the value of this reaction in view of the nonspecificity. It appears that the plate test, and under certain conditions (specific serum inhibitions or studies with pure cultures of known species designation) the tube reaction, may prove of considerable value. The reactions listed above have been obtained constantly and with a sufficient number (when available) of strains of the given species as to leave little doubt that they may be considered typical.

It is true that certain species give similar plate reactions—group I: *C. perfringens*, *C. sorelli–C. bifermentans*, *C. hemolyticum*, and *C. novyi* type B; group II: *C. parabotulinum* types A and B and *C. botulinum* type B; and group III: *C. novyi* type A and types C, D, and E of *C. botulinum*. This does not, in our opinion, destroy the value of the reaction; because if the species designation of an unknown strain is narrowed to the members of a given group, the differentiation of the species comprising the group would be easy by physiological reactions and in some instances almost unnecessary if the origin of the strain in question is known. In addition, the following characters on the plate medium are of value in the recognition of the named species. The reaction of *C. novyi* type B may be distinguished from others in group I by the clearly defined edge of the precipitation zone. On standing, the outer extra zone is separated from the original by a heavy narrow area of precipitate. The colony of *C. hemolyticum* is more raised, more regular, and smaller than those of the other species in relation to the reaction zone. The colonies of the *C. sordelli* group tend to be flatter and more transparent and irregular than the usual smooth type of *C. perfringens* colony. The reactions of *C. sordelli*, on the whole, seem to be wider than those of the nontoxic *C. bifermentans*, but, within the group, some strains of

C. sordelli give zones that are narrower than the widest zone of *C. bifermentans.* Within group II, the colonies of *C. botulinum* type B tend to be less raised and the reaction zones relatively wider than those of *C. parabotulinum.* The colonies of *C. botulinum* type B resemble those of the other *C. botulinum* types, even though the reaction is very much like that of *C. parabotulinum.* The luster area of the types C, D, and E of *C. botulinum* is narrow and follows the contour of the flat, irregular colony. In the typical *C. novyi* type A reaction, the luster area is wide and circular. A few strains of *C. novyi* showed narrower luster zones, which followed the colony edge and closely resembled the reactions of the *C. botulinum* types.

DISCUSSION

The egg yolk agar plate reaction would appear to be of considerable value as a presumptive species reaction in clinical and other laboratories. In the food research laboratory the differentiation of *C. sporogenes* and *C. parabotulinum* is difficult, and the reactions described above may be of great value in the early recognition of *C. parabotulinum* from samples involved in botulism. In the clinical laboratoty it appears possible that the reaction will be of aid in the rapid identification of certain of the gas gangrene organisms. Although it is possible that the reactions may be elicited by colonies obtained by streaking directly from wound exudates, it may be necessary to enrich such samples in suitable liquid media such as Brewer's thioglycolate broth and to inoculate egg yolk agar plates after 4 to 6 hours of incubation.

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SUMMARY

A peptone base medium and an egg yolk supplement are described for use in plate culture demonstration of the LV (lecithovitellin) or Nagler reaction. By use of this medium presumptive identification of the following is possible: *Clostridium perfringens* (*C. welchii*), *C. novyi* (*C. oedematiens*), *C. sordelli–C. bifermentans*, *C. hemolyticum*, *C. botulinum*, *C. parabotulinum*, and *C. sporogenes.*

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ULTRAVIOLET IRRADIATION OF BACTERIOPHAGE DURING INTRACELLULAR GROWTH

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In the course of attempts to produce mutations in bacteriophage by ultraviolet irradiation of bacteria infected with the phage, we found that suppression of the ability of an infected bacterium to liberate phage required higher doses than either sterilization of noninfected bacteria or inactivation of free phage. The doses required to suppress phage liberation varied in the course of the interval between infection and liberation, during which intracellular phage growth takes place. These observations were in agreement with the fact that phage multiplication can take place in bacteria recently sterilized by irradiation (Anderson, 1944; Rouyer and Latarjet, 1946). They suggested that the observed effect of radiation on the infected bacteria might depend on inactivation of intracellular phage. Variations of this effect might then reflect the changes in number and properties of phage particles during intracellular growth—that is, in the course of processes that lead to the production of over 100 phage particles from each infected bacterium (Delbrück, 1946). An analysis of the changes in ultraviolet sensitivity during the period of intracellular growth could then be expected to supply information on the mechanism of growth.

The rate of ultraviolet inactivation of free phage is a simple exponential function of the dose of radiation (one-hit inactivation, see Latarjet and Wahl, 1945). If inactivation of the individual intracellular phage particles followed the same function, and if the ability of a bacterium to liberate phage depended on the survival in it of at least one active particle, then the number of intracellular particles per bacterium at the time of irradiation should influence the rate of suppression of phage liberation. Instead of an exponential one-hit inactivation curve, as for free phage, we should find for the infected bacteria a multiple-hit curve, the number of hits reflecting the number of active particles present at the time of irradiation.

The feasibility of this analysis was suggested by some preliminary observations of this type by Anderson (1944). His data, which he kindly discussed with us, seemed to indicate a shift from one-hit to multiple-hit type of curve in the inactivation curves for phage-infected bacteria during intracellular phage growth.

The process of intracellular phage growth—in particular, of the kinetics of phage production—has so far escaped every attempt at clarification made either by breaking down infected bacteria or by electron microscopy. This problem is of such fundamental interest that we considered it worth while to explore thoroughly the new lead which ultraviolet irradiation seemed to offer. The results of this investigation are discussed in the present paper.

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MATERIAL AND TECHNIQUE

Escherichia coli, strain B, and bacteriophage T2 were used. Some experiments with bacteriophage T7 and with *Escherichia coli*, strain B/r, were also done. Bacteria and phages were grown in an ammonium glucose phosphate buffer medium with salt complements, chosen for its transparence to ultraviolet light. In this medium *Escherichia coli* B grows with a generation time of 40 minutes at 37 C; phage T2, in the presence of a standard young culture of B, has a latent period of 21 minutes between infection and the beginning of liberation; liberation is complete 35 to 40 minutes after infection; the average phage yield per bacterium is 100 to 150. Phage T7 has a latent period of 13 to 14 minutes, complete liberation before 25 minutes, and a yield of 40 to 60. The values for time of liberation and phage yield are less reproducible in our medium than in nutrient broth.

The experimental technique for irradiation of growing phage is as follows: All suspensions are maintained in a water bath at 37 C. The desired amount of phage lysate (titer measured by plaque count) is introduced into an aerated bacterial culture containing 5 to 10 $\times$ 10^7 bacteria per ml, as assayed by viable count. After allowing a definite short time for phage adsorption (generally 30 to 60 seconds), a sample of the mixture is diluted in a solution of antiphage serum capable of inactivating in 30 to 60 seconds all the remaining free phage. This does not affect the course of phage growth inside the infected bacteria (Delbrück, 1945*b*). A further heavy dilution in serum-free medium, to give a serum concentration without appreciable absorption of ultraviolet, yields a suspension of bacteria part or all of which are infected by phage. The proportion of infected bacteria and the average number of phage particles adsorbed per bacterium can easily be regulated by varying the initial proportions of phage and bacteria. When only a fraction of the bacteria is infected, each infected bacterium will adsorb only one phage particle ("single infection"). When most or all bacteria adsorb several phage particles, we speak of "multiple infection."

The number of infected bacteria is measured by plaque count, by plating a sample on agar with an excess of sensitive bacteria. Each infected cell gives one plaque up to the time when phage liberation begins. The infected bacteria, as numbered by plaque count during the latent period, will hereafter be defined as "infective centers."

Samples of the final highly diluted suspension of infective centers are taken at intervals and exposed to radiation within 45 seconds from the time of sampling. The time of irradiation is always given as the time when exposure begins, although some exposures lasted as long as 80 seconds. The irradiated samples are immediately assayed to determine the proportion of infective centers still capable of producing plaques. When the phage yield is to be determined, the samples are quickly returned to a temperature of 37 C and assayed at intervals, a nonirradiated sample which has undergone the same manipulations as the irradiated ones being used as a control.

The source of radiation was a General Electric germicidal lamp, of the low-pressure type; 80 per cent of its ultraviolet output consisted of wave length 2,537 A. As this radiation is especially efficient in bacterial sterilization and phage inactivation (Gates, 1934), more than 95 per cent of the effects were due to it. We could therefore consider our ultraviolet beam as almost monochromatic. Its intensity was measured by comparison with the beam from a similar lamp calibrated in absolute units by Dr. A. Hollaender, and it was recalibrated at frequent intervals.

In most experiments the samples were exposed at a distance of 56 cm from the bulb and received a uniform flux of 16 ergs $\times$ mm^{-2} $\times$ sec^{-1} for the wave length 2,537 A. Variations in intensity, when desired, were obtained by varying the distance from the bulb, the dependence of intensity upon distance having been determined by suitable photoelectric measurements.

The samples were irradiated in open dishes 5 cm in diameter. The depth of the suspensions was about 1 mm, the bacterial concentration not higher than 10^4 per ml. Under such conditions, no mutual screening of bacteria took place, and the dose was uniform throughout the sample.

RESULTS

Killing of noninfected bacteria and of free phage.[3] The survival curve of B in synthetic medium was determined down to a survival of 10^{-4}, using clear, nonscreening suspensions. The results (curve 1, figure 1) show that the killing is an exponential function of the dose. As observed by Witkin (1946), a change in the slope of the curve appears for survivals less than 10^{-2}, as if 1 per cent of the bacteria had higher resistance.

The survival curve of T2 was determined down to a survival of 10^{-5}, and proved to be a regular exponential function of the dose (curve 2, figure 1). Our results agree to within 2 per cent with those previously obtained in Paris by Latarjet and Wahl (1945) using a calibrated beam of 2,537 A. This agreement shows the reliability of spectrophotometric ultraviolet measurements in absolute units and the value of phage inactivation as a biological test for estimating the intensity of monochromatic ultraviolet light. The survival of the more resistant phage T7 is given in curve 4 of figure 1.

Evidence that killing of infective centers is due to killing of intracellular phage. This evidence was brought out by several observations. First, if killing of an infective center results from direct action on the phage, the killing curve for single-infected bacteria immediately after infection, before any growth takes place, will be very similar to that of free phage. The only expected difference will be a slight increase in resistance due to possible screening of the phage particle by a layer of bacterial protoplasm. The killing curve for B single-infected with T2, two minutes after infection (curve 3, figure 1), is actually very similar to that of free phage. That this is not a fortuitous coincidence is shown by the existence of the same relation for the more resistant phage T7 (curve 5, figure 1).[4]

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[3] The term "killing" is used for convenience throughout this paper. The more precise expression would be, in the case of bacteria, "sterilization"; in the case of free phage, "inactivation"; and in the case of infected bacteria, "loss of ability to liberate active phage."

Further evidence was obtained by using, instead of strain B, a mutant strain B/r, derived from B and much more resistant than B to radiation (Witkin, 1946).

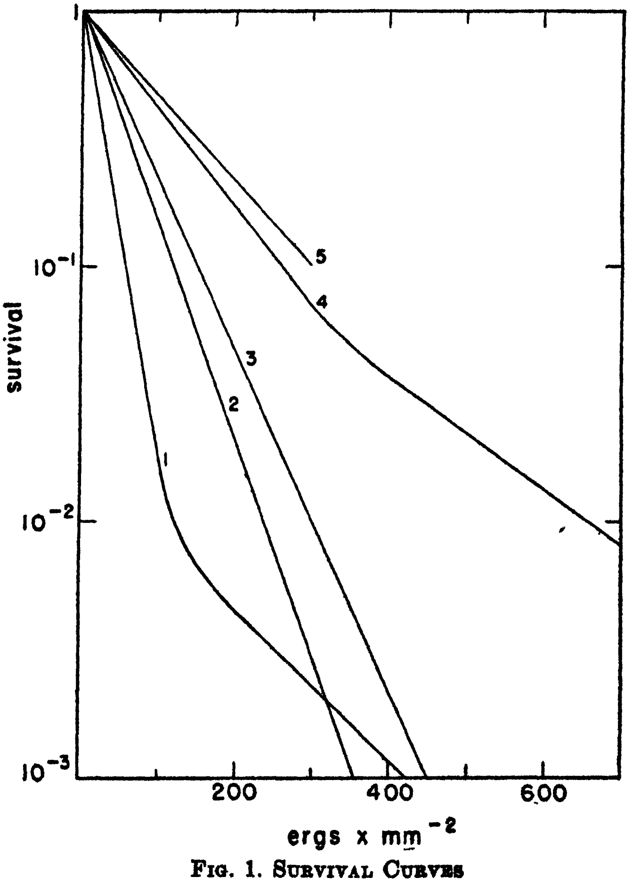

FIG. 1. SURVIVAL CURVES

(1) *Escherichia coli*, strain B. (2) Phage T2. (3) T2 infective centers, 2 minutes after single infection. (4) Phage T7. (5) T7 infective centers, 3 minutes after single infection.

The infective centers for T2 still showed the same resistance. The radiosensitivity of the bacterium itself, therefore, does not seem to determine the sensitivity of the infective centers.

Moreover, as will be seen later, multiple infection leads to "multiple-hit" survival curves for the infective centers, with multiplicity closely corresponding to the average number of phage particles adsorbed per bacterium. We can conclude, therefore, that killing of infective centers results from killing of intracellular phage, at least at the beginning of the latent period.

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[4] Further experiments with phage T7, similar to those described in the following sections for phage T2, were hampered by technical difficulties, mainly concerned with the use of concentrated antiphage serum, and will not be discussed in this paper.

Survival curves of infective centers in the case of single infection. Figure 2 shows the results of a typical experiment in which one out of every two or three bacteria was infected with one T2 phage particle. The suspension of infective centers was irradiated at various times (2.5, 4, 6, 8, 10, 12, 15, and 18[illegible]minutes) after infection. At each time three doses (320, 640, and 1,280 ergs × mm^{-2}) were given in 20, 40, and 80 seconds, respectively. The curves give, in semilogarithmic co-ordinates, the survival of infective centers as a function of the dose at each time. They illustrate the regularity of the results within one experiment.

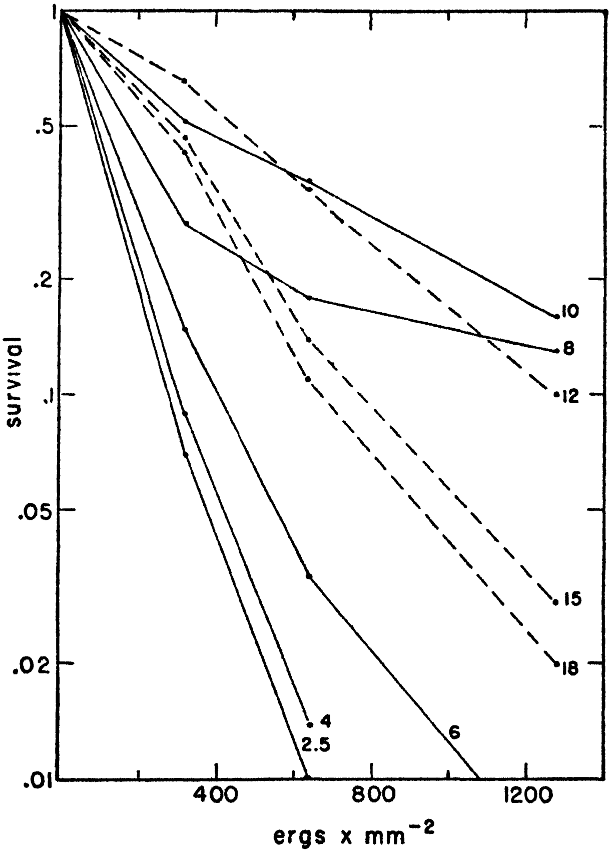

FIG. 2. SURVIVAL CURVES OF T2 INFECTIVE CENTERS AT VARIOUS TIMES

Data from a single experiment. The numbers indicate the time of irradiation (minutes after infection).

A large number of comparable experiments were performed, in which the proportion of infected bacteria was between ½ and $\frac{1}{10}$.[illegible]Doses from 50 to

1,180 ergs × mm^{-2} were given at various times. Altogether, 242 experimental values were thus collected, covering almost every minute of the latent period for a variety of doses. Values for the same dose given at the same time displayed some degree of variability, and were averaged graphically. The averages were used to construct the set of curves in figure 3, (a) and (b), in which survival is plotted in logarithmic scale as a function of the dose. The results can be described as follows:

The resistance of infective centers to radiation increases progressively during the first part of the latent period, up to 11 minutes. Early in this period (at 3 and 4 minutes) the survival curves are almost exponential, showing a slight increase in resistance with time. From 5 minutes on, however, survival for high doses increases more rapidly than for low doses, so that the successive curves show increasing amounts of upward concavity. From 7 minutes on, a slight downward concavity appears in the upper part of the curves, making them deviate more and more from simple exponentials.

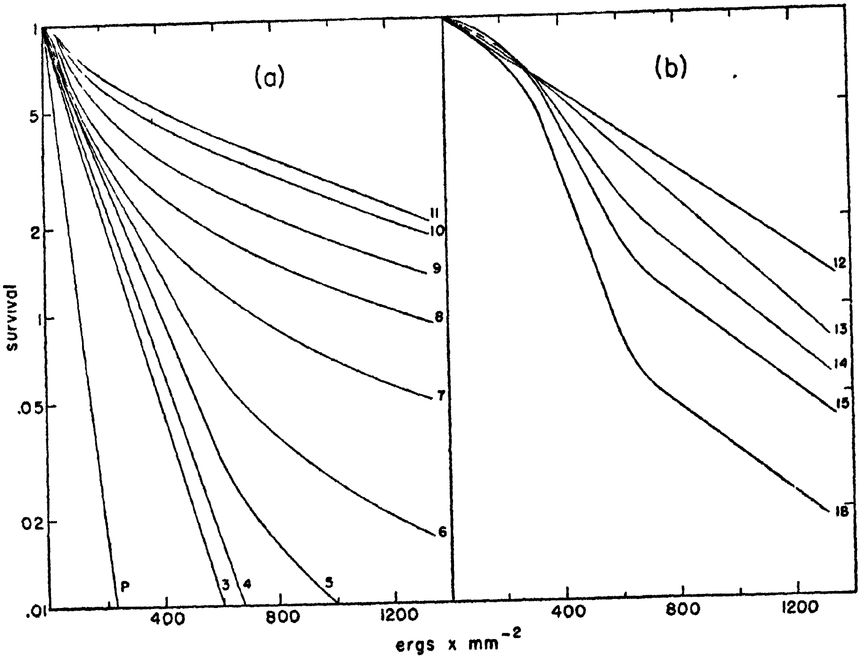

FIG. 3. SURVIVAL CURVES OF T2 INFECTIVE CENTERS

Averages of all the data from experiments with single infection, 1 out of 2 to 10 bacteria being infected. (a) Early times; (b) late times. The numbers indicate the time of irradiation. Curve *P* is the survival curve for free phage.

After 11 to 12 minutes the general trend changes. Although the downward concavity for low doses becomes more pronounced, resistance to high doses drops progressively with time, until after 15 to 18 minutes the survival curves become more and more of the multiple-hit type (see figure 5). A discussion of these curves will be given in a later section.

Dependence of the survival curve for single infection on the proportion of infected bacteria. In the preceding experiments, in which 1 out of 2 to 10 bacteria was infected, the fluctuations of individual results were slight and nonsystematic. Significant deviations appeared, however, in experiments in which we infected only 1 out of 25 to 150 bacteria by introducing smaller amounts of T2 lysate into the bacterial culture. It is important to remember that in both cases we have single infection of practically all of the infected bacteria. The probability that any one bacterium will adsorb two or more phage particles is very low whenever the average number of particles adsorbed per bacterium is much less than one. Length of the latent period and yield of phage per bacterium are the same in both cases.

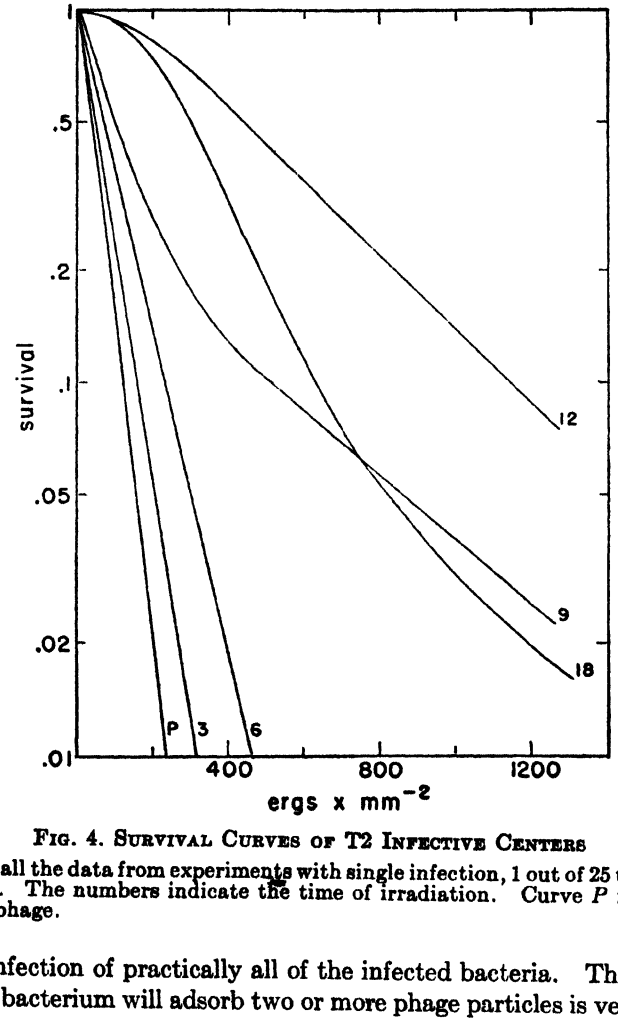

FIG. 4. SURVIVAL CURVES OF T2 INFECTIVE CENTERS

Averages of all the data from experiments with single infection, 1 out of 25 to 150 bacteria being infected. The numbers indicate the time of irradiation. Curve *P* is the survival curve for free phage.

Numerous experiments, performed with low proportions of infection, gave consistent results. Altogether, 153 experimental values were collected and averaged; the survival curves are given in figure 4. Comparison of figures 3 and 4 shows the following facts:

Both sets of curves show the same trends, with an early increase in resistance and, for high doses, a late decrease. Moreover, from 12 minutes on, the curves in the two sets are practically identical. Striking differences are present, however, in the survival curves for early times. When the proportion of infected bacteria is low, the sensitivity to radiation in the first minutes is greater, and the increase in resistance progresses more slowly. It appears that some process responsible for the changes is delayed. For example, the survival curve for 6 minutes is still practically exponential, similar to that for 4 minutes in figure 3. The delay is compensated for by a swift increase after 6 to 7 minutes, so that by 12 minutes no difference remains.

What causes the more rapid increase in resistance in cases with a high proportion of infected bacteria? Experiments under a variety of conditions excluded the possibility of any influence of either the concentration of antiserum or the time during which the bacteria were exposed to it. Since experiments with low and high proportions of infection were comparable in every respect except for the amount of phage lysate introduced, we must conclude that the difference between the two cases is due to the presence in the lysate, and adsorption by the bacteria, of some other material besides the active phage. With larger amounts of lysate, enough of the unknown active material is probably introduced to exert its effect on part or all of the infected cells and change the rate of increase in resistance. The same phenomenon was found for all the lysates of phage T2 tested.

As the survival curves are the same for proportions of infection between 1:150 and 1:25, it is likely that in these cases the changes in resistance during the latent period are due to the action of the active phage only.

Phage yield from irradiated infective centers. In a number of experiments we measured the yield of phage from the infected bacteria irradiated at various times with different doses. Irradiation at any time produces a definite decrease in yield, between 20 and 50 per cent. No systematic dependence on the dose or the time of irradiation was found during most of the latent period. Infected bacteria irradiated at 15 minutes or later, however, yield only 10 to 20 per cent as much phage as the control. No appreciable delay in phage liberation is caused by the irradiation.

These results seem to indicate that, after irradiation, phage growth can continue in those bacteria where some active phage remains. If irradiation takes place very late, the surviving phage will have less time left to grow, and the yield will accordingly be smaller.

Multiple infection. We have presented evidence that ultraviolet killing of infective centers results from inactivation of the intracellular phage. The survival curve for single infection at very early times is close to the exponential curve for the survival of free phage. In the case of multiple infection each bacterium adsorbs several phage particles. If only one of these particles could penetrate the cell and grow (mutual exclusion, Delbrück, 1945c) the survival curves would be similar to those for single infection. If, however, several particles can grow in the same bacterium, the survival curves will be affected.

The survival function for single particles is $y = e^{-aD}$, where y is the fractional survival, D the dose, and α a constant defining sensitivity. If there are n active particles per bacterium, all with the same sensitivity, and if an infected bacterium is counted as one infective center as long as one phage particle at least remains active, the survival function of the infective centers will be

$$y = 1 - (1 - e^{-\alpha D})^n. \tag{1}$$

The corresponding set of curves for various values of n is given in figure 5.[5]

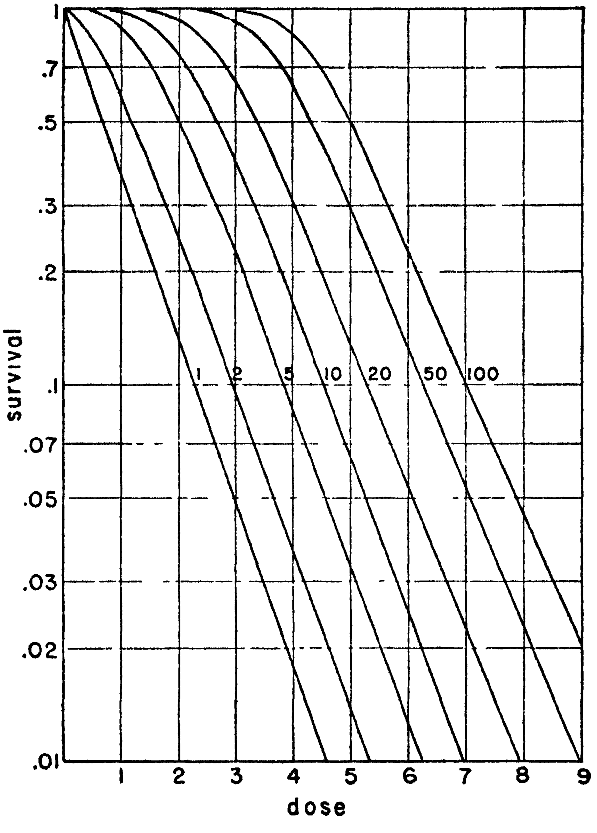

FIG. 5. THEORETICAL SURVIVAL CURVES CORRESPONDING TO FORMULA (1)

Dose in arbitrary units. The numbers on the curves refer to the corresponding values of n in the formula.

When we irradiated bacteria multiple-infected with phage T2, we found that from the earliest times (4 minutes after infection) the survival curves were very

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[5] The actual multiplicity of infection must vary from cell to cell within a culture around the average value n. Assuming a Poisson distribution, Dr. M. Delbrück derived the following expression:

$$y' = 1 - e^{-ne^{-}} \tag{1'}$$

The suvival curves calculated according to formula (1′) are very similar to those calculated from formula (1) and shown in figure 5.

different from those obtained for single infection, and of a definite "multiple-hit" type. Several experiments were performed, giving, in all, 96 consistent values. These could not be averaged, as in the case of single infection, because the multiplicity of infection varied from one experiment to another. Figure 6 shows the results of some individual experiments.

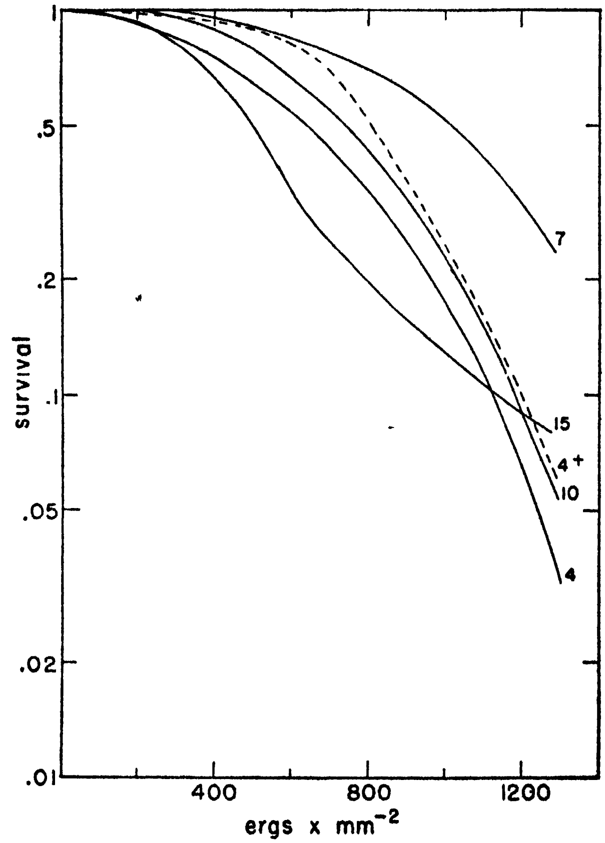

FIG. 6. SURVIVAL CURVES OF T2 INFECTIVE CENTERS, MULTIPLE INFECTION

Each curve represents a separate experiment. The solid lines refer to experiments with multiplicity of about 5 phages per bacterium. The broken line (+) refers to an experiment with a multiplicity of about 15. The numbers on the curves indicate the time of irradiation.

A comparison with the theoretical curves of figure 5 shows that the shape of the experimental curves for early times is similar to that of the theoretical curves for values of *n* corresponding to the multiplicity of infection. An increase in multiplicity of infection causes a shift in the curve toward higher values of *n*. For example, the shape of the curve for 4 minutes, multiplicity 5, is similar to that of the theoretical curves for 4 to 5 hits, whereas the curve for 4 minutes, multiplicity 15, is more similar to a 15- or 20-hit curve. The multiplicity of infection is necessarily a roughly estimated quantity and may vary rather widely from cell to cell in the same culture. Better agreement with theoretical curves is scarcely to be expected. The results clearly indicate, however, that most or all of the infecting phage particles remain active inside the bacterium and can participate in the process of growth.

The survival curves for multiple infection at 4 minutes (figure 6), compared with those for single infection (figures 3 and 4), show resistance higher than can be explained on the basis of the multiplicity alone. For example, at 4 minutes, for multiplicity of 5, the dose leaving 50 per cent survival is 750 ergs × mm^{-2} as compared with 85 ergs × mm^{-2} for single infection at the same time. The ratio of the doses is 9. Comparison of the curves for $n = 1$ and $n = 5$ in figure 5 shows a theoretical ratio of 3. The discrepancy indicates that the resistance of the individual particles in the case of multiple infection is higher than in single infection. This is probably due to the fact that in experiments with multiple infection the bacteria come in contact with higher concentrations of phage lysate—that is, with greater amounts of the unknown component which, as previously discussed, plays a role in increasing the resistance of intracellular phage.

In experiments with multiple infection the resistance of the infective centers increases fast, reaches a maximum between 6 and 7 minutes (instead of 11 to 12 minutes as for single infection), then decreases. Thus it seems that for multiple infection the growth process arrives earlier at the stage at which the trend of the changes in resistance is reversed.

The magnitude of the increase in resistance between 4 and 7 minutes, while the survival curve maintains its multiple-hit shape, seems to indicate that all the infecting particles actually participate in the growth process, rather than that one particle grows and the others are capable only of replacing it if it should be inactivated.

The decrease in resistance late in the latent period makes the survival curves at 15 minutes and later resemble closely those for single infection. This agrees with the known fact that the end results of phage growth are the same for single and multiple infection, as regards both duration of the latent period and phage yield per bacterium (Delbrück and Luria, 1942).

DISCUSSION

What information concerning the mechanism of phage growth can be derived from these experiments? We shall first consider some of the possible events in the growth processes and see how our results agree with the corresponding expectations.

In the case of single infection, we could suppose that in every bacterium phage multiplies at the same rate, producing identical particles capable of further multiplication. We have seen that in newly infected bacteria radiation acts by direct inactivation of intracellular phage. If the survival of an infective center after irradiation depended on the survival of at least one active particle, the survival curves should become multiple-hit curves, similar to the theoretical curves of figure 5, corresponding to progressively higher values of n as growth proceeds. The curves for successive times would reveal the number of active particles per bacterium at each time. Figures 3 and 4 clearly show that the process of phage growth does not fit this simple picture.

Let us consider what happens in the case of single infection during the first 11 to 12 minutes. The main change is a progressive increase in the resistance of infective centers to radiation. The survival curves show a progressively increasing upward concavity (figures 3 and 4), the opposite of what would be given by any multiple-hit phenomenon. We must, however, keep in mind that whatever phage multiplication takes place inside the bacteria is likely to proceed at varying rates, so that at any one time there must be bacteria with widely different numbers of particles. In fact, Delbrück (1945*a*) observed a wide distribution of the phage yields from individual infected bacteria. It was thought that the survival curve might reflect the variability in phage multiplication. We tried, therefore, to calculate a distribution of the number of phage particles that would account for our experimental curves. This calculation led to very improbable assumptions. In particular, the upward concavity of the curves could only be explained by extremely bimodal distributions. The survival curve at 9 minutes (figure 4), for instance, would require a mixture of 80 to 90 per cent cells containing 1 to 5 particles and 10 to 20 per cent containing 50 to 100 particles per cell.

Since high doses require a longer time of exposure (up to 80 seconds), it was thought that the upward concavity of the curves might be due to the fact that part of the dose was received when phage growth had already reached a later stage. This possibility was ruled out by experiments with high intensity and short exposures, which gave the same results.

We consider as the most likely interpretation that during the early part of the latent period most of the change in the survival curves is caused not by phage multiplication, but by a progressive increase in the resistance of the individual particles. A change in the ultraviolet sensitivity of phage particles during intracellular growth is a novel feature, but hardly a surprising one if we think of the complicated processes that must take place inside the infected cell.

The simplest mechanism by which the increase in resistance could be brought about would be the accumulation, around the particles, of some ultraviolet-absorbing material. Let us consider, for example, the curves for 3 and 10 minutes in figure 3. The doses giving 50 per cent survival are 70 and 290 ergs $\times$ mm^{-2} respectively (ratio = 4). A survival of 20 per cent results from doses of 190 and 1,220 ergs $\times$ mm^{-2}, respectively (ratio = 6.4). If no phage multiplication took place, this increase in resistance of about 4 to 6 times—the variation being due to the different shape of the survival curves—could be explained by the accumulation between 3 and 10 minutes of enough material around the phage to absorb 4 to 6 times more radiation. Such an increase in resistance could be provided, for example, by a layer of about 200 mμ of a substance having an extinction coefficient of 30,000 cm^{-1}, the approximate value for nucleic acids. If some phage multiplication takes place, it will in itself produce some increase in resistance; the amount of screening material required will be less than calculated above. Indeed, the slight deformation of the initial part of the survival curves after 7 to 8 minutes (figure 4) is probably an indication that some multiplication has started by this time.

According to the "screening" hypothesis, the change in sensitivity of the intracellular phage would be apparent rather than real. This hypothesis may be too naïve, however. It is easy to imagine other mechanisms that may affect the intrinsic sensitivity of the phage particle. Phage inactivation may, for example, be caused by absorption of radiation in any one of a number of chemical structures within the particle, and the growing phage may contain fewer of these "vital spots." Also, the probability that absorption in a certain structure results in inactivation may vary during growth as a result of changes in chemical reactivity. There exists strong evidence (Pirie, 1946) that the state of virus particles inside the host cell may actually be quite different from that of free particles.

In the following discussion we adopt the hypothesis of accumulation of absorbent materials. Most of the considerations would still be valid if changes in sensitivity resulted from any of the other mechanisms discussed above.

The upper concavity of the survival curves is probably due to large fluctuations in the rate of evolution of the growth process in different cells. These fluctuations are in agreement, and possibly in causal relation, with the previously mentioned variability in phage yield per bacterium.

The dependence of the resistance of the infective centers on the absolute amount of phage lysate with which the bacteria have been in contact has indicated that some other component of the lysates, besides the active phage, influences the early rise in resistance. We have no information yet about the nature of this component. Studies on its sedimentation in the ultracentrifuge, its heat resistance, and other properties may clarify its nature.

Sensitivity of the infective centers to high doses of radiation begins to increase 12 minutes after infection for single-infected bacteria and 8 minutes for multiple-infected ones. Since the sensitivity to low doses does not increase, the survival curves become more and more of the multiple-hit type. We can imagine that, as the end of the latent period approaches, phage multiplication takes place in most bacteria, while the screening material disappears, possibly being used up in phage reproduction. These two processes could account qualitatively for the shape of the survival curves at late times.

It is also likely that the presence of many particles inactivated by radiation may interfere with the ability of the remaining ones to carry the process of phage liberation to its successful completion. Thus, the apparent increase in sensitivity at late times may be partly due to failure to count as infective centers some bacteria still containing active phage but unable to liberate it.

On the basis of our results we may propose a very tentative and probably crude picture of the sequence of events in intracellular growth of phage T2. After one or more phage particles have penetrated into a bacterial cell, an ultraviolet-absorbing material, possibly needed for phage building, accumulates and intercepts part of the incident radiation. Additional stimulus for the accumulation of this material can be supplied by some other component present in phage lysates besides the active phage itself. The accumulation of screening material seems to vary widely from cell to cell, and this variation may be partly responsible for the variability of phage multiplication in different cells, reflected in the variability of phage yield. Phage multiplication is apparently under way 7 minutes after infection, and as it proceeds the amount of screening material around the phage particles begins to diminish. In the last part of the latent period most bacteria probably contain large numbers of particles. The distribution of these numbers is probably connected, not only with the distribution of burst sizes, but also with the variability of the time of lysis for individual cells.

This picture seems to be in agreement with some preliminary results of cytological studies of the growth of phage T2 (Luria and Palmer, unpublished). The first reaction to phage infection is seen as a disruption of the Giemsa- and Feulgen-positive "nuclear" bodies (see Robinow, 1945) and migration of their material toward the periphery of the cell. The amount of stainable material in the cell then increases rapidly until the whole cell, somewhat enlarged, becomes stained in a fine granular way. If this "nuclear" material is assumed to contain a large proportion of nucleic acid, the amounts of it visible in stained preparations are more than sufficient to account for the increase in apparent phage resistance during growth.

The apparent changes in sensitivity of the individual phage particles during the latent period make it impossible to attempt calculation of the rate of phage multiplication from the results of ultraviolet irradiation of infective centers. If our interpretation of the changes in phage sensitivity as due to protection by nonphage material is correct, it might be possible to avoid this difficulty by using X-rays instead of ultraviolet light. Even for X-rays, however, the analysis of the survival curves may be rendered very complicated by the large fluctuations expected in the rate of phage multiplication within individual cells.

The results of our experiments with multiple infection indicate growth of most or all of the infecting particles. The principle of mutual exclusion, according to which only one of several particles adsorbed by the same cell can grow, seems not to hold for particles of the same phage strain. Evidence to the contrary, derived from experiments on interference between a phage and one of its mutants (Luria, 1945), was not too conclusive, in view of the fact that infection with particles of the two types was not simultaneous, and deserves reconsideration.

It appears that mutual exclusion always takes place between particles of unrelated phages (Delbrück and Luria, 1942; Delbruck, 1945*c*), is somewhat limited between particles of related phages (Delbrück and Bailey, 1946), and does not occur between particles of the same strain. The similarity of the yield of phage in cases of single and multiple infection is likely to be due, not to mutual exclusion, but to the amount or rate of formation of some substrate which limits phage multiplication.

SUMMARY

Ultraviolet irradiation of *Escherichia coli*, strain B, infected with bacteriophage T2 showed that, immediately after infection, suppression of the ability to liberate phage results from inactivation of the intracellular phage.

The sensitivity of the infected bacteria was studied during the 21-minute interval between infection and lysis. In the first 12 minutes, the infected bacteria show a rapid increase in resistance, apparently due to increased resistance of the intracellular phage particles. This is possibly caused by accumulation of ultraviolet-absorbing material around the phage. At later times the resistance of infected bacteria to high doses of radiation decreases. This is interpreted to indicate that, as phage multiplication proceeds, the apparent sensitivity of the intracellular phage particles returns to higher values.

A quantitative study of phage multiplication by an analysis of the survival curves of infected bacteria is made impossible by these changes in sensitivity of the individual phage particles during growth, and by the presence of wide fluctuations, in the course of phage growth, among individual infected cells.

Phage lysates appear to contain, besides the active phage itself, some other component which influences the course of the intracellular phage growth as manifested in the changes in ultraviolet sensitivity described above.

In case of infection of a bacterial cell with more than one particle of phage T2, analysis of the survival curves shows that several particles can grow in the same host cell.

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THE EFFECTS OF X-RAYS ON A STRAIN OF EBERTHELLA TYPHOSA

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The study of the effect of X-rays on bacteria is suggested from the success which geneticists have had in using X-rays on multicellular forms as a means of inducing genetic changes. If it may be postulated that bacteria have the same genetic setup as higher forms, then it is conceivable that bacteria might be affected in the same way by X-rays. This would follow along the same line of work as Muller (1927), who produced mutations in many types of reproductive and somatic cells with the use of X-rays. The present report is concerned with the effect of X-rays on a strain of *Eberthella typhosa.*

Most of the literature concerning the effect of X-rays on bacteria relates only to the lethal action. Progress has been reviewed at intervals by Clark (1934), Duggar (1936), McCulloch (1945), and Rahn (1945). There is evidence to indicate that there is a distinct difference between killing a bacterium by X-rays and killing a bacterium by heat or by disinfectants. Thus, according to Lea (1946), "after irradiation, the bacterium which is rendered incapable of giving rise to a colony may still be motile (Bruynoghe and Mund, 1935), may still be capable of respiration (Bonet-Maury, Perault and Erichsen, 1944) and may, when cultured and examined microscopically, show some growth (Luria, 1939)."[1] Thus, the killing action of X-rays on bacteria is possibly the result of the production of a lethal mutation. Whether bacteria have the same hereditary mechanism as higher plants is still a matter of conjecture, but it is quite probable that they do. Thus, according to Lewis (1941), a nucleus of a bacterium might be considered as "a naked gene string encrusted with chromatin, or a single naked gene string." If the bacterium is a haploid (which is most probable since it is asexually produced), then there is a great possibility of a lethal mutation having immediate effect.

There have been few publications concerning the changes that may occur in X-rayed cultures. The first to study the effect of X-rays on bacteria was Minch (1896). He exposed a strain of *E. typhosa* on agar to the X-rays produced from a Hittorf tube at a distance of 10 cm for a period of 8 hours. His only observation was that there appeared to be no gross change in the development of the colony. Haberland and Klein (1921) exposed a human strain of the tubercle bacillus to X-rays and found no change in its biochemical or physiological activities. On the other hand, Lange and Fraenkel (1923) noted that the infectivity for guinea pigs was greatly diminished when they used a 33-day-old X-rayed culture of the human type of *Mycobacterium tuberculosis.* They demonstrated that younger cultures were more resistant to the X-rays. Klovekorn (1925) exposed *Escherichia coli* and *Staphylococcus aureus* to X-rays and noted that there were certain modifications in the cultural characteristics only when the cultures were 28 to 30 days old. Bertrand (1929) found that X-rays of 2 A had no effect on the virulence or rapidity of growth of *S. aureus* or *Microsporon audouini*. Rice and Guilford (1931) showed that X-ray treatment of a bovine strain of *M. tuberculosis* increased the dissociation from "rough" to "smooth" colonies in a rapidly growing culture.

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[1] The references given in this quotation are not specifically cited in this paper.

Smith, Lissc, and Davey (1936) noted that there was no significant change in the electrophoretic mobility of *Escherichia coli* after exposure to X-rays. Forfota and Hâmori (1937) claim that the antigenic structure of *E. typhosa* undergoes change under the effect of hard X-rays. Lea, Haines, and Coulson (1937) found that occasionally long filamentous rods would be formed from an X-rayed culture of *E. coli*. This was explained as due to the interference with the fission mechanism. Drea (1938, 1940) showed that the virulence of a culture of human tubercle bacilli could be considerably attenuated by successive irradiations with X-rays over a long period of time.

Haberman and Ellsworth (1940) noted the increase in dissociation of *S. aureus* and *Serratia marcescens* in actively proliferating cells. Their work seems to indicate that hard rays were more effective in producing dissociants. Haberman (1941) showed that a culture of staphylococci exposed to X-rays lost the lethal factors, and those of dermoneurosis and hemolysis. There was no observable association in colony types. It appears from the work of Lea, Haines, and Bretscher (1941) that X-rays of various lengths on *E. coli* and spores of *Bacillus mesentericus* produced lethal mutations. The striking effect was that the bacteria continued to grow, in the sense of increasing in size, but failed to divide. Gray and Tatum (1944) produced, by means of X-rays, mutant strains of *E. coli* and *Acetobacter melanogenum* characterized by their inability to produce specific biochemical reactions. Tatum (1945) likewise produced mutant strains of *E. coli* by exposing two mutant strains to a second X-ray treatment.

Some of the effects of X-rays upon bacteria which have been noted are the production of lethal action, permanent changes in colony form and color, and the loss of certain biochemical reactions.

MATERIALS AND METHODS

X-ray apparatus. A General Electric crystal diffraction X-ray unit, type VWC, form E, was used. It was operated at 30,000 volts with a filament current of 18 milliamperes. A molybdenum target Coolidge type with unfiltered radiation was used. The maximum characteristic radiation was 0.712 A. These rays were hard, but, because of the presence of continuous radiation and the use of unfiltered radiation, some soft rays were present.

In general, the rays are spoken of as hard or soft on the basis of their wave length. Those which have wave lengths greater than 1 A are classified as soft rays and those which have wave lengths less than 1 A as hard rays. The soft rays are more readily absorbed by living material, but the hard rays are more penetrable.

The bacteria that were exposed to the rays from this machine were placed at a distance of 6⅜ inches from the X-ray tube in front of the X-ray window. The bacteria, in a distilled water suspension, were placed in a small pyrex glass tube and were accurately placed in the path of the X-rays by means of a fluorescent screen directly behind the tubes.

Culture. The strain of *Eberthella typhosa* used had been recently isolated from the blood of a typhoid patient. The strain was characteristic of the species in respect to all the biochemical and physiological characteristics as described in Bergey's *Manual* (1939). Neither its antigenic formula nor its phage specificity was determined.

For the purpose of this study a single cell of this culture was isolated and put into nutrient broth. It was incubated at 37 C for 18 hours and was streaked on agar slants. These agar slants were incubated for 18 hours, and the growth was harvested by means of gentle washing with sterile, distilled water. The suspension was then diluted with sterile, distilled water to give a concentration by the nephelometer method of McFarland the no. 10 tube, which corresponds to about 3 billion organisms per ml as determined by plate counts. One ml of this suspension was then placed in a chemically clean, sterile pyrex glass test tube. The suspension was then ready for exposure.

Lethal studies. The tubes containing the suspension of the organisms were held securely in a clamp against the window of the X-ray machine. The organisms were exposed for periods of ½, 1, 1½, 2, 2½, 3, 3½, 4, and 4½ hours.

Immediately after exposure, the suspension was diluted in a tenfold series to a dilution of 1:1,000,000,000. Sterile distilled water in 9-ml amounts was used for the dilutions, and nutrient agar pour plates were made in duplicate from each of the dilutions. All plate counts were made after an incubation period of 48 hours. Only those plates that contained from 30 to 300 colonies per plate were used to determine the number of bacteria that survived. A control consisting of 1 ml of the same suspension that was unexposed to the X-rays was used in all cases. The temperature during exposure was 25 C.

It was found that the killing of the organisms resulted in a logarithmic order of death. The results were plotted on semilogarithmic paper (figure 1). After ½-hour exposure, 50 per cent of the organisms had been killed; at the period of 4 hours, only 0.05 per cent survived; and at 4½ hours, none survived.

It has been shown by several workers (Lea, 1946) that what is described as a lethal effect of radiation upon a bacterium is the inability to give rise to a colony on appropiate laboratory media. The bacterium may still be capable of performing some of its life processes. Thus it was of interest to observe whether or not the actively motile culture of *E. typhosa* was still motile after 4½ hours of irradiation. After this period of exposure the organisms were examined for motility by the hanging drop method. The great majority of bacteria appeared to be nonmotile, but a small percentage showed motility. However, when these organisms were placed in suitable media, they failed to develop colonies.

Anomalous variation. The modifications of bacteria have been called mutations, variations, dissociations, saltations, discontinuous variations, etc. There is little real justification for the use of these terms as applied to bacteria.

Our conceptions of heredity have been derived from living things which pass through a sexual cycle and which have a nuclear mechanism. We do not have adequate knowledge concerning the hereditary mechanism of bacteria to interpret them in similar terms.

The views concerning the existence of nuclei in bacteria range from the supposition that bacteria have no nuclei to the view that the entire cell is a nucleus (Lewis, 1941). We may postulate that the bacterium is a haploid since it is asexually produced, but it may be a diploid or even a polyploid. Even when we are dealing with a single bacterial cell, we cannot be sure that we are dealing with only one nuclear unit (Wilson and Miles, 1946).

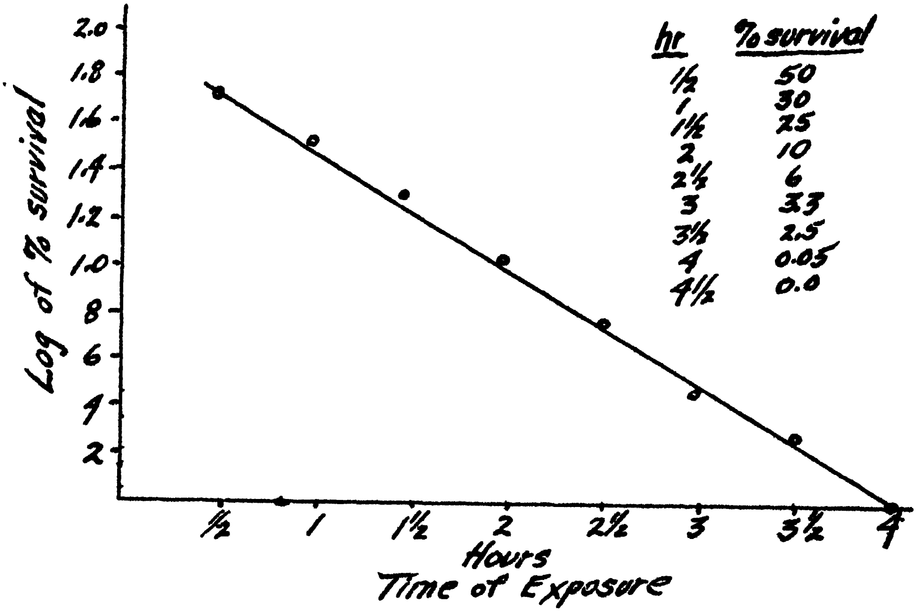

FIG. 1. THE EFFECTS OF VARIOUS EXPOSURE TIMES ON THE SURVIVAL RATES OF EBERTHELLA TYPHOSA

Bacteria may also be considered as premitotic. Their hereditary constitution might thus be conceived as not being differentiated into specialized functions and parts. Thus the hereditary processes in bacteria may be quite different from those of multicellular organisms. According to Huxley (1942), "One guess may be hazarded; that the specificity of their constitution is maintained by a purely chemical equilibrium, without any of the mechanical control superposed by the mitotic (and meiotic) arrangements of higher forms." He implies that it is unreasonable to expect bacteria to have fully developed mitotic mechanisms. The mitotic mechanism of higher organisms is a complicated, highly developed process. It must have resulted from long and gradual evolution. It seems reasonable to assume that bacteria as a group may exhibit numerous steps in the evolution of the mitotic process. Some may be entirely premitotic and depend upon some quite unknown mechanism for the transmission of hereditary tendencies. Others may have partly developed or fairly well developed mitotic mechanism.

Since it is desirable to avoid any implication concerning the hereditary mechanism of bacteria (until we have adequate knowledge), the term *anomalous variation* is suggested. *Anomalous* connotes the idea of a deviation from normal order that refuses to submit to an explanation or classification. Variations in organisms are known to be due to the following causes: (1) changes in the environment, (2) gene mutation, (3) changes in chromosome complexes, (4) gene recombination or hybridization, and (5) a combination of any of these four. A sixth category may be added for convenience to include all causes in which the causative mechanism of the variation is unknown, namely, *anomalous variation*. It is noted that this is a classification based on causes of variation. Thus, for example, the modification of a bacterial culture that may occur after exposure to X-rays is an anomalous variation, as well as any other variation the causative mechanism of which is unexplained at the present time.

The plates used above in determining the number of organisms killed also served as material for the study of anomalous variation immediately after X-ray treatment. Ten exposures to X-rays were made at different times, and the colonies surviving the 4-hour period of exposure were studied by means of a colony microscope lens (3 ×)to note any changes in morphology. At random 100 colonies that survived this period of exposure were picked each time. Approximately 0.05 per cent of the organisms had survived this length of time. A total of 1,000 colonies picked were transferred to semisolid agar to determine motility, by the method of Tittsler and Sandholzer (1936), and incubated for 24 hours. After this period of time transfers were made to various other media and studied from the following aspects: colonial character on agar and gelatin; morphology and stain by Gram's method; fermentation of lactose, glucose, and xylose; lead acetate production; indole formation; growth on potato; and motility as indicated by flagella staining.

The colony morphology of the 1,000 colonies observed remained similar to the parent unexposed control culture. Likewise, all the other tests remained the same as the control, with one exception. After one exposure, from which 100 colonies were picked at random, 78 of the colonies demonstrated a loss of motility. The colony morphology of the 78 nonmotile forms was the same as that of the unexposed control colonies when first examined. Subsequent plating of these cultures, however, showed a change in the colony formation from a smooth to an intermediate form. All the other tests used concerning these 78 nonmotile forms remained the same as the control. The 78 nonmotile cultures were, in addition, tested in the following substances: raffinose, galactose, maltose, fructose, salicin, sorbitol, sucrose, dulcitol, inulin, inositol, and mannitol. The fermentation reactions remained the same as the unexposed parent control culture.

In addition to these anomalous variation studies, suspensions in distilled water of an 18-hour culture of *E. typhosa* were exposed to the X-rays in exactly the same manner as described for the lethal studies. But, in addition to the same time periods of exposure, the organisms were irradiated for 1, 5, 10, 15, 20, 25, 30, and 35 minutes. After exposure, 0.5 ml of the bacterial suspension were transferred to 9 ml of nutrient broth and incubated at 37 C. Subcultures on agar plates were made daily for a period of 20 days from the 15 cultures, as well as from the unexposed control.

Observations were made on the colonial character of the organisms after 48-hour incubation. The colony form was the only observation made since this type of variation would be easily detected. However, this character of the exposed cultures remained the same as that of the parent unexposed type. This anomalous variation is not the only one that might have occurred, and it is quite possible that other changes were overlooked.

DISCUSSION

The culture of *E. typhosa* used in this study was isolated first from the blood of a typhoid patient and then by single cell technique. Observations of the parent unexposed culture did not show any detectable changes in the colonial character. Approximately 3,000 unexposed colonies were observed.

The lethal studies confirmed the findings of other investigators and showed that the rate of death is of a logarithmic order. The interesting observation is that there is a distinct difference between killing a bacterium by radiation and, for example, by heat. Some of the bacteria were still motile after 4½ hours of exposure, although they failed to produce any growth on suitable laboratory media.

The anomalous variation observed was the loss of motility in 78 out of 100 colonies picked at random after one of the experimental exposures. This loss of motility was not observed again even after many repeated X-ray exposures.

The observations made on the X-rayed cultures were limited to a few morphological and biochemical reactions. It is quite possible that other changes may have occurred but were not observed.

ACKNOWLEDGMENT

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SUMMARY

A strain of *Eberthella typhosa*, isolated from the blood of a typhoid patient and then isolated by single cell technique, was exposed to X-rays of maximum characteristic radiation of 0.712 A.

The so-called "lethal" effect of the X-rays resulted in a logarithmic order of death. Some of the organisms, however, were still motile but failed to grow on suitable laboratory media.

After 4 hours of exposure, in which 0.05 per cent survived, the culture was

studied from the following aspects: colonial character on agar and gelatin; morphology and stain by Gram's method; fermentation of lactose, glucose, and xylose; lead acetate production; indole formation; growth on potato; and motility. All the results of these tests on the organisms exposed to X-rays remained the same as those of the tests on the unexposed control, with one exception. On one occasion, a high percentage of the organisms exposed showed a loss of motility. These nonmotile forms were in addition tested in other sugars, but the fermentation reactions remained the same as in the control.

Subsequent plating of these nonmotile cultures showed a change in colony formation from a smooth to an intermediate form.

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INTRACELLULAR BACTEROIDS IN THE COCKROACH (PERIPLANETA AMERICANA LINN.)[1]

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Problems involving the so-called "intracellular symbionts"[2] have long troubled some cytologists and bacteriologically inclined zoologists. The solution of many of those problems, however, yet evade even the most careful investigators. Several workers in this field (Buchner, Glaser, Mercier, Schwartz) hold to the view that the physiology and systematic position of the intracellular bacteroids can be accurately determined only by the cultivation of these organisms on artificial media. Toward this end, many workers (see Schwartz, 1935) have struggled with sterilization and dissection techniques and experimented with various culture media in what has usually been futile or unsatisfactory attempts to grow the bacteroids in unnatural conditions.

The extracellular "symbionts" in the gut of insects are generally not strict in their growth requirements (Schwartz, 1935; Steinhaus, 1941), and some of the forms that have an intracellular stage in the gut wall and an extracellular stage in the gut lumen seem to grow readily on artificial media from their extracellular stage: e.g., those of species of *Sitodrepa*, *Ernobius*, *Rhagium*, *Xestobium* (Heitz, 1927; Muller, 1934), *Rhodnius* (Wigglesworth, 1936), and others (see Schwartz, 1935, p. 398). The more closely adapted intracellular organisms, however, grow with difficulty if at all, and none of the reports of successful cultures of these are above serious criticism on matters of sterilization and manipulation.

The intracellular bacteroids of the cockroaches, with which this paper deals specifically, have been so thoroughly described (Fraenkel, 1921; Gier, 1936; Bode, 1936; Hoover, 1945) that it suffices here to say that these bacteroids are nonmotile, nonsporeforming, faintly gram-positive, straight or slightly curved rods, 0.8-1 μ by 1.5-6.5 μ, and may stain barred, somewhat like diphtheroids. They occur around the ovarian eggs and in specialized cells, the mycetocytes of the abdominal fat bodies.

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[1] The work reported here was begun at Indiana University under the direction of Dr. Fernandus Payne, as part of a doctorate problem, and was continued at Harvard University Biological Laboratories on a National Research Council Fellowship and a Harvard University Research Fellowship under the general supervision of Dr. L. R. Cleveland and Dr. A. B. Dawson. Aid and counsel which made this work possible and profitable are gratefully acknowledged.

[2] The term "symbiont" definitely connotes a helpful association between two types of organisms. Such a relationship has never been demonstrated for any of the true "intracellular symbionts." Mercier (1907) designated the bodies of the roach mycetocytes as "bacteroidi" and this lead was followed by Hertig (1921), Gier (1936), and Hoover (1945). It seems better thus openly to confess our ignorance as to the nature of these bodies by continuing to designate them merely as "bacterialike" than to imply a relationship which probably does not exist, even though these "bacteroidi" may sometime be definitely placed taxonomically with the bacteria.

There have been alternate reports of success and failure in attempts at cultivating these bacteroids since the early failures of Blochmann (1887), Krassiltschik (1889), and Forbes (1892). Mercier (1907), Glaser (1920, 1930), Gropengiesser (1925), and Bode (1936) reported success, but Javelly (1914), Hertig (1921), Wollman (1926), and Hovasse (1930) admitted failure. These efforts have recently been summarized by Buchner (1930) and Steinhaus (1940).

Mercier (1907) cultivated, from the ootheca of *Blatta orientalis*, a motile, sporeforming bacillus which he named *Bacillus cuenoti*. These cultures had the effect of dispelling permanently the idea championed by Cuenot, Prenant, and Henneguy that the "symbionts" of the roaches and other insects were only metabolic products Mercier's work was discredited by the failure of Javelly (1914) and Glaser (1920) to cultivate *Bacillus cuenoti*. Hertig (1921), in turn, showed quite definitely that the spirillum cultivated by Glaser (1920) was not the "symbiont." Gropengiesser (1925) and Bode (1936), however, cultivated motile, sporeforming rods from *Blatta orientalis* and *Periplaneta americana*, respectively, which they concluded were identical with *B cuenoti* in spite of certain discrepancies in the published descriptions. Mercier (1907) and Gropengiesser (1925) also frequently cultivated a yeast that they believed was a secondary "symbiont" that could, on occasion, displace the bacteroids, but neither gave any evidence for his contention Glaser (1930), in a series of very carefully executed experiments, cultivated three strains of diphtheroids from *Periplaneta americana* and attempted to prove seriologically that they were the "symbionts." More recently, Hoover (1945) has reported successful cultivation of diphtheroids and other bacilli from *Cryptocercus*.

In view of these conflicting results, it seemed desirable to check critically the various techniques and media used in past cultivation experiments, to try new methods, and to attempt to analyze results more thoroughly.

The first difficulty, and the source of the most constant error in such cultivation experiments, is the problem of securing the "symbiotic" organism from the host tissue without contaminating the material with bacteria that may subsequently be mistaken for the "symbiont." The ideal way to eliminate contaminants is to rear the insects aseptically from previously sterilized eggs. Wollman (1926) and Bode (1936) developed techniques for doing this with *Blattella germanica* and *Periplaneta americana*, respectively, but both failed in culturing any bacteria from such sterile roaches. The most convenient method of obtaining uncontaminated, "symbiont-laden" material is the sterilization of the oothecae chemically, using the contents of the oothecae directly as inoculation material. The third and most treacherous method is the chemical sterilization of the exterior of the roach and the removal of the "symbiont-laden" tissues. Glaser (1920, 1930), Hertig (1921), Gropengiesser (1925), and Bode (1936), have developed fairly satisfactory techniques along these lines.

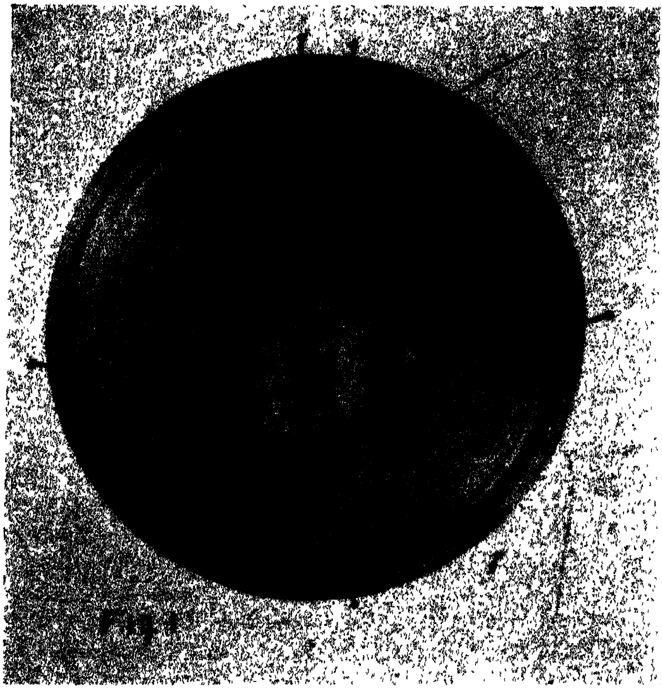
The second and possibly the greatest difficulty in such cultivation experiments is the provision of adequate culture media for the "symbionts." Since the physiological and chemical properties of the natural habitat of these organisms are incompletely known, an adequate medium can be found only by the trial and error method. It would be expected that organisms as highly specialized as the intracellular "symbionts" would require a very special medium. Mercier (1907) and Gropengiesser (1925), however, cultivated *Bacillus cuenoti* readily on most routine bacteriological media. Schwartz (1924) used a general medium with high sugar content for the yeastlike "symbionts" of the *Lecanidae*. Meyers (1925) used routine beef extract peptone media enriched with an extract of snails on which to cultivate the "symbionts" of the concretion organs of certain snails. Glaser (1930) depended on blood media for the cultivation of the roach symbionts, and Hoover (1945) followed Glaser's techniques. All these workers reported successful cultures with their respective methods, but their results have not been confirmed.

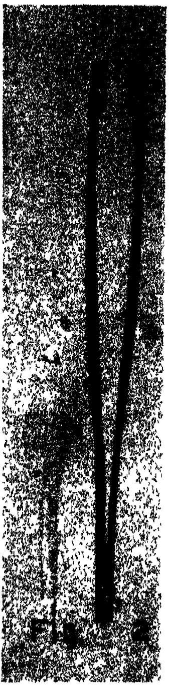
The third, and probably most perplexing, problem in "symbiont" cultivation lies in the identification of the cultivated organism. In the past, morphologic similarity between the cultivated form and the intracellular form plus dependence on the adequacy of the technique used have been the main criteria, and these, as will be shown later, are not reliable. Glaser (1930) attempted identification by serological comparisons, which to date has not been developed to reliability.

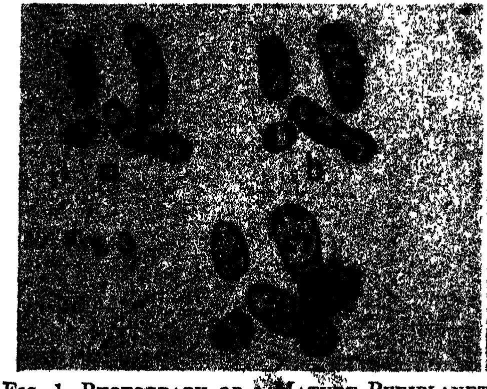
MATERIALS AND METHODS

For the following series of experiments, the American cockroach, *Periplaneta americana* (Linnaeus), was used most extensively as the source of inoculation material, being supplemented at times with *Blatta orientalis* (Linnaeus), *Parcoblatta pennsylvanica* (De Geer), and *Cryptocercus punctulatus* Scudder. All forms except the last were successfully reared in the laboratory (Gier, 1936, 1946).

Sterilization, dissection, and inoculation techniques were modified from those described by Hertig (1921) and Glaser (1930). Nymphs and adults to be used for bacteriological work were kept on clean filter paper in glass bowls, without food, for several days, so they would be as clean as possible and have little material in the gut. On removal from the bowl, each animal was pressed lightly to remove fecal pellets. Sterilization and dissection were conducted as follows: the roach was etherized until completely immobile, dipped into 95 per cent alcohol, agitated for 5 minutes in a solution consisting of equal parts of 1:500 mercuric chloride and 95 per cent alcohol, then rinsed in 70 per cent alcohol. The animal was then placed on its back in a dish of freshly melted and solidified paraffin, and secured with pins: one through the edge of the prothorax, one through the tip of the abdomen, and one on each side of the body posterior to the metathoracic legs crossing over the body and holding the legs forward well out of the way (figure 1). Then with a pair of fine scissors all the abdominal sternites, except the last, were cut along their left margins; the sternites were carefully grasped by their free margin with sterile forceps and the entire ventral body wall, as a unit, was turned over to the right and secured there with one or two pins. With fine forceps portions of the fat body or the ovary from the right side (side opposite the cut) were removed, separated from trachea and Malpighian







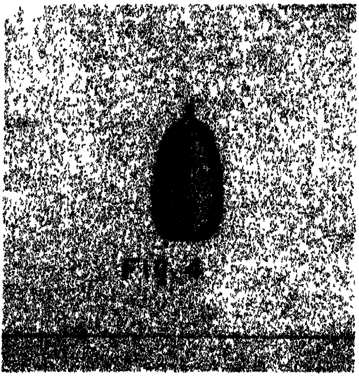


Fig. 1. Photograph of a Mature Periplaneta americana, Showing Method of Securing the Roach for Removal of Tissues

Materials for bacteriological examination were always taken from the side opposite the incision, thus precluding any possibility of external contamination. (Natural size)

Fig. 2. Forceps Used for Holding the Ootheca for Removal of Embryos Aseptically

The depression at the tip is of such a size that it holds the capsule firmly, yet without danger of crushing. (Natural size)

Fig. 3. A Clump of Roach Bacteroids Showing Progressive Stages of Vacuolization in Hypotonic Solution

The stage shown as *C* is possibly the condition described by Mercier and Cuenot as yeast that had invaded the mycetomes and displaced the normal bacteroids. (X 2,000)

Fig. 4. Section of Ootheca to Show Relationships of Embryos, Mucus Layer, and the Fused Edge of the Capsule. (X 2)

tubules in sterile Belar's solution, and transferred to the media in which they were macerated.

When eggs or embryos were to be used as the source of the inoculum, special precautions were taken to get the best possible oothecae. In order to keep a plentiful supply of embryos, vigorous adult females were kept in clean glass bowls (250-mm biological specimen dishes of the type that will stack) with plenty of food and water. The oothecae were taken from the females as soon as they were complete, which was usually 12 to 18 hours after their appearance from the vagina, and were stored in clean watch glasses. Sterilization and dissection techniques were the simplest possible. A perfectly formed, clean, unshriveled ootheca of the age desired was selected from the stock; it was dipped into 95 per cent alcohol, placed in the mercuric chloride, alcohol solution for 15 minutes, and rinsed in 70 per cent alcohol. One end of the ootheca was then grasped firmly with special forceps (figure 2) and the other end sliced off with a red-hot razor blade and discarded. The two eggs thus exposed were removed with a hot inoculating needle and discarded; the remaining eggs were transferred directly to the media, or they were macerated in the capsule with a sterile inoculating loop before the transfer was made.

All instruments were sterilized either in open flame or boiled in 70 per cent alcohol. The sterilizing fluids were freshly boiled and cooled. Sterile pins and other small instruments were all handled with sterile forceps. Dissections and inoculations were done in a tightly closed room that had been scrubbed the previous afternoon and sprayed well with dilute phenol solution shortly before the work was begun. As an added precaution against air contaminants, the last series of experiments was done under a sterile hood, and the bare arms of the worker were sterilized with mercuric chloride, alcohol mixture.

The efficiency of these methods needs little comment other than to point out that few contaminations occurred, as is shown in table 1. Absolute sterilization of either cockroach or ootheca is probably impossible because of rather frequent infections in the oviducts, Malpighian tubules, trachea, or hemocoele. The time necessary for the application of the sterilizing fluids was determined, after much experimenting, as that sufficient to perfect external sterilization, and yet not enough to damage the animals seriously. Cockroaches that were allowed to revive after sterilization lived normally; and embryos in about 80 per cent of unopened, sterilized oothecae continued normal development.

The media used included Petroff's egg medium, Loeffler's coagulated blood serum (both horse and calf serum), deep brain media, potato, nutrient gelatin, beef extract broth with agar, Hutoon's hormone broth with agar, and modifications especially of the latter two. Beef extract and hormone broths were used as the basis for blood media (2 to 20 per cent defibrinated blood from horse, cow, or human added), and roach extract media (1 to 10 per cent extract from roaches added). This roach extract was made by either boiling, macerating, and filtering, or by digesting with trypsin, quantities of roaches, usually with alimentary canal removed, and was sterilized by the Berkefeld filter, inspissator, or autoclave. Inoculations were made both in liquid media and on solid media, the latter being preferred because of better isolation of contaminants. Petri plates were inoculated by spreading macerated eggs, embryos, or fat bodies over surface of the media in four or five consecutive spots, which were left separated by a few millimeters' space only (Glaser, 1930). This gave a dilution so great that rarely did any growth occur on the last two spots. In many cases a second plate was inoculated without reinfecting the loop, in order to check Glaser's (1930) theory that the symbionts will grow only if their natural inhibiting agents are greatly diluted.

The reaction of the media used was varied from pH 6.4 to pH 7.8 to cover the complete range of findings of hydrogen ion concentration in insect blood (Glaser, 1925; Bodine, 1926; and others), but most were adjusted to pH 7.2 because of my own findings on the hydrogen ion concentration in *Periplaneta americana* blood, on the assumption that the pH of protoplasm is the same as that of the surrounding blood.

The pH of the blood was determined colorimetrically and checked with a Gesel quinhydrone electrode. A drop of phenol red indicator was placed on an opal plate, one antennae of the roach clipped off, and the blood run directly into the indicator. Readings taken immediately were invariably 7.1 to 7.3, rising within 2 minutes to 7.4 to 7.5. For the electrometric check, blood was drawn into the Gesel chamber directly from the cut antennae and a reading taken as quickly as possible. The result was 7.3 ± 0.1 when one animal supplied sufficient blood, or 7.4 ± 0.1 if the blood of two animals was used. Tests made after 10 minutes or more invariably ranged between 7.55 and 7.65. The lower hydrogen ion concentration in the latter case is probably due to the loss of carbon dioxide and cannot be considered as normal. Samples of blood were diluted 1:16 with water without changing the pH more than 0.1 point, indicating a very efficient buffer action. Crushed cells in the test solution invariably increased the acidity to pH 5.8 to 6.8.

The salt content of the media was varied from 0 to 1 per cent, and the osmotic pressure was further increased at times by the addition of sugars, urea, sodium acid phosphate, potassium sulphate, and other salts in an attempt to make the media isotonic with the cockroach blood, which was found to depress the freezing point approximately 0.9 C as against 0.62 C for horse blood, which indicates a much higher concentration of salts in the roach blood.

All media were incubated 3 days at 30 C before they were inoculated, and all plates or tubes showing any contamination were discarded. About 200 cultures were tried under anaerobic conditions as stabs, shakes, tubes sealed with oil, Kumwiede-Pratt plates, and Novy jar cultures with the oxygen completely or partially displaced with carbon dioxide or the oxygen removed with pyrogallol. Most cultures were incubated at 30 C, as that was found to be the optimum temperature for *Periplaneta americana* (Gier, 1946). Others were incubated at room temperature or at 36 C. The inoculated media were examined daily for growths, and everything but obvious contamination, i.e., colonies between the inoculated spots, was carefully checked. Inoculated spots showing no growth after 3 or 4 days were carefully rubbed up with a drop of condensation fluid as described by Glaser (1930), and material was transferred to slants of the same kind of media. These subcultures were examined, and the slant surface was flooded with condensation fluid daily for at least 10 days. If the agar became dry, a few drops of serum broth were added (Glaser, 1930). On the fourth or fifth day of incubation, whether or not a macroscopic growth could be seen, new transplants were made, and slides were prepared, stained, and examined microscopically from each original subculture.

RESULTS

Series I. This series of approximately 500 culture attempts was conducted at Indiana University. Materials for inoculation were taken in about equal numbers from *Periplaneta americana*, *Blatta orientalis*, and *Parcoblatta pennsylvanica*. The technique and media used were in general those described above, with emphasis on no one kind of medium. The results of this series were not at all convincing because of the high incidence of positive cultures on plates (about 35 per cent of all plates showed growths) and the great variety of organisms in these cultures. Most of these positive cultures were readily identified as contaminations by direct correlation of the cultivated organisms with bacteria occurring commonly in the environment of the roach. With each refinement of technique, however, the incidence of positive cultures declined so that, before work on this series was terminated, the sporeforming rods comparable to *Bacillus cuenoti* were of rare occurrence. Six cultures of diphtheroids, which were not readily explained as contaminations, were isolated in this series by subculturing apparently sterile spots. These were very similar to the diphtheroids described by Glaser (1930).

TABLE 1

Attempts at cultivating the intracellular symbionts of Periplaneta americana and Cryptocercus punctulatus

(Series II; see text for explanation)

SOURCE OF INOCULUM	NO. OF TRIALS	PLATES CONTAMINATED			ORGANISMS PRESENT				
		Heavily	Slightly	None	Yeasts	Bacilli	Cocci	Sarcinae	Diphtheroids
Oothecae	93	9	7	77	5	8	3	4	2
Ovaries	39	3	3	33	1	4	2	2	1
Fat bodies	69	6	3	60	0	5	3	2	0
Total	201	18	13	170	6	17	8	8	3
Water (controls)	10	3	1	9	0	1	0	0	0

Series II. This series, conducted at Harvard University, was for the most part a duplication of series I except for greater refinements in technique and in the use of *Periplaneta americana* as the source of inoculum, supplemented with *Cryptocercus punctulatus*. Dissections were done on 15 days, at intervals of approximately 2 weeks. Twelve to 15 plates, plus controls, were inoculated on each dissection day.

The results are given in table 1. Of the 201 plates inoculated, 170 remained apparently sterile for at least 3 days. Growths on the 30 plates were, for convenience, designated as heavy contaminations, with a general heavy growth over any part of the plate, or as slight contaminations, with a few isolated colonies affecting only one or two spots. The organisms growing on these plates were of many different kinds, including a number of molds not listed in table 1, but all were found frequently as air contaminants on control plates or in cultures from the gut and from the exterior of the roach. Besides the contaminations listed, there were a total of 20 contaminating colonies on the plates definitely off the inoculated areas.

Of the 782 subcultures (table 2) from apparently sterile spots, only 28, or 3.58 per cent, showed any bacterial growth within the 10 days the cultures were kept under observation. These 28 positive cultures were of at least 12 different kinds of organisms: i.e., at least two kinds of yeasts, four kinds of diphtheroids, three kinds of other bacilli, two kinds of staphylococci, and one *Sarcina*.

TABLE 2

Results from subculturing apparently sterile spots of plates listed in table 1

ORIGINAL SOURCE OF INOCULUM	TRANSPLANTS	ORGANISMS ON TRANSPLANTS				
		Yeast	Bacilli	Cocci	Sarcinae	Diphtheroids
Oothecae	384	10	2	0	2	5
Ovaries	144	1	1	1	0	3
Fat bodies	254	0	1	0	2	2
Totals	782	11	4	1	4	10
Water (controls)	41	0	0	0	0	0

Series III. A number of attempts to grow the "symbionts" *in vivo* was conducted after the failure of bacteriological cultures became evident. In one experiment 25 hen eggs were incubated at 37 C for 8 to 10 days. A small triangular window was cut through the egg shell, and a roach embryo or clump of fat body was implanted on the chorioallantois, with adequate precautions against contamination. The hole in the shell was sealed with paraffin, and the egg was returned to the incubator at 35 C. Temperatures higher than this were usually fatal for roach embryos, and hence would probably be unfavorable for the bacteroids. Sixteen of the chick embryos lived until the eggs were reopened 5 to 7 days later. In most cases the roach tissue was walled off and was in the process of being absorbed; in two, the inoculum was not located, and in three others, the roach embryo apparently provoked no reaction from the chick and both continued normal development to the end of the experiment. In another experiment, suspensions of bacteroid-bearing fat bodies were injected with a capillary pipette into the amniotic cavities of five 7-day-old chick embryos. On the fourteenth day, the eggs were reopened, but only degenerating bacteroids were found.

Series IV. Many attempts were made to grow the bacteroids in tissue culture, both in tubes and in hanging drops. Fat body clumps kept in drops of roach blood gradually lost their bacteroids during a period of about 2 weeks. The way in which the bacteroid number decreased was not definitely determined. Fat body clumps and bacteroid-laden portions of embryos lived in apparently normal condition for as long as 3 weeks in small tubes of media consisting of peptone meat extract broth 7 parts, horse blood serum 2 parts, and 10 per cent glucose solution 1 part, reaction adjusted to pH 7.0 to 7.2. In these cultures there was no indication of bacteroid growth, although about 1 out of 8 showed contaminating bacterial growth. Sixty hanging drop cultures of embryonic tissues, fat bodies, or gonads in roach blood, crayfish blood, cricket blood, horse serum broth, or chick amniotic fluid showed no signs of bacteroid growth although some of these were maintained, with two transplants, as long as 3 weeks.

Various and numerous controls were run concurrently with the inoculation experiments as follows:

Air controls. At the time of each series of dissections, a plate of the medium used for inoculation was left open on the table or under the hood for 30 minutes. A number of different kinds of bacteria found on the inoculated plates were found also in these air controls.

Inoculation technique controls. During the course of each series of inoculations, one plate was spotted in the regular fashion, using as inoculum sterile Belar's solution such as was used for washing the roach tissues. In 10 such plates, only one colony, a gram-negative rod, appeared.

Sterilization technique controls. Frequently plates or tubes of media were inoculated with non-bacteroid-bearing parts of the roach body, as a leg, a segment of muscle, a portion of the ventral body wall, or a clump of Malpighian tubules. Positive cultures of various sorts occurred in approximately 1 out of 10 from muscle. Inoculations with large clumps of fat body showed nearly the same incidence of infection and the same bacteria as did inoculations with Malpighian tubules—20 per cent positive cultures. Oothecae placed in broth after they had been sterilized and the embryos removed produced positive growths, usually of yeasts and sarcinae, in about one case in four. The same organisms were obtained in the same relative frequency by inoculating with only the lips of the sterilized ootheca clipped off beyond the tips of the eggs, showing conclusively that most of the contaminations from oothecae come from between the lips where chemicals do not reach them and where they are not normally disturbed by the dissection technique used in this work; but the contamination would be transferred if the ootheca was opened along the seam or macerated. Numerous tubes of media (nutrient agar slats, broth, and gelatin stabs) were inoculated with bacteroid-laden and non-bacteroid-laden tissues. In these, positive cultures were relatively less frequent than with plates because chance for air contaminants was reduced to a minimum, but the same contaminants were encountered. Broth cultures were extremely unsatisfactory because of the uncertainty of the original quantity of contaminant.

A considerable number of sterilized oothecae were placed on sterile agar slants until the nymphs emerged. The aseptic nymphs were reared for as long as four months on sterile nutrient agar slants, with whole-wheat flour, yeast extract, and blood added. Attempts were made to cultivate the symbionts from these aseptic nymphs, but no bacterial growth appeared on any medium used in the 25 trials.

DISCUSSION AND SPECIAL CONSIDERATIONS

Several times during the course of this work, I felt certain that I had at last cultivated the roach bacteroid. First, there was an unidentified gram-negative, nonsporulating rod that occurred in nearly 50 per cent of the inoculated plates for several dissections, but after a change of cages and food supply for the animals, the prevalent bacteria suddenly became *Serratia marcescens*. After a considerable refinement of technique, the prevalent bacteria, occurring in nearly 10 per cent of inoculations, was a gram-positive, sporeforming rod that answered the description of *Bacillus cuenoti* as well, at least, as did the organism cultivated by Gropengiesser (1925). With greater precautions in sterilization and dissection of roaches and oothecae, however, the occurrence of this sporeforming rod was gradually reduced to very infrequent intervals, no matter what medium was used. Obviously, then, this sporeforming rod was not the bacteroid, or it would grow regardless of more careful manipulation.

As the technique was refined to eliminate the counterpart of *Bacillus cuenoti*, some very slow-growing diphtheroids occurred on subcultures from apparently sterile spots, usually on blood media. Usually they appeared about the same time on the plate from which the subculture was made. These diphtheroids fitted the description of Glaser's *Corynebacterium periplaneta* very well: they were barred, gram-positive; sometimes pleomorphic; did not liquefy gelatine; utilized glucose, sucrose, and maltose without gas formation; grew slowly at first, doing well only on blood media, and gradually became adapted to routine culture media. These bacteria never occurred in more than 6 per cent of the subcultures from any set of inoculations, and sometimes 30 plates were inculated and the regular 120 subcultures made from them with no organisms appearing except a stray yeast, a sarcina, or a slow-growing gram-negative bacillus. Strangely enough, the fewest bacterial growths appeared in the cultures from the series of dissections done under the most nearly optimum conditions.

The explanation for the diphtheroid cultures came accidentally one day when an air-control plate of blood medium was being examined under the microscope for the preliminary identification of colonies. On that plate were found two very tiny, nearly transparent droplets, only about 0.1 mm in diamter. They had the appearance of the diphtheroid colonies that had been studied and actually proved to be such. Similar diphtheroid colonies were found on nearly every air-control plate used after that. More numerous colonies and more kinds of diphtheroids were obtained by planting plates of Glaser's blood media in secluded spots in various offices in the Harvard Biological Laboratories. Other diphtheroids were isolated from the exterior and from the gut of the cockroach in cultures that were not too quickly covered by spreading colonies of more hardy forms. Numerous cultural tests failed to differentiate the diphtheroids of the experimental cultures from those of the air-control cultures, so the only conclusion that can be drawn is that the diphtheroids in the experimental cultures are contaminants that are peculiarly favored by the blood media and spotting technique used by Glaser. These diphtheroids, that seem to be everywhere, produce such tiny colonics in the original culture that it is almost impossible to distinguish them from fat droplets on the inoculated area, but when they are transferred to fresh media, they make enough growth in a few days to be readily visible. They probably enter the plates in the first place as air contaminants, because occasionally numbers of colonies of these diphtheroids were found on control plates left open under the supposedly sterile hood. The spotting technique, which necessitates opening the plate several times, is especially favorable to such airborne contaminants, and the procedure of subculturing apparently sterile spots makes visible these otherwise unnoticed colonies.

No attempt has been made to explain the presence, cultural behavior, or taxonomic position of these diphtheroids since it is quite evident that they are not the bacteroids of the cockroach. Some of them, however, were culturally indistinguishable from *Corynebacterium periplaneta*, Glaser.

In the results of these cultivation experiments, one fact is emphasized: that by any one special technique, one or a few kinds of bacteria are favored, resulting in partial or total elimination of other types of contaminants.

Glaser (1930) attempted to prove that the diphtheroids cultivated by him were identical with the "symbionts" by injecting suspensions of the diphtheroids into living roaches. Heavy suspensions of the diphtheroids did not kill them; therefore, he concluded, the roach must have a special natural immunity to this diphtheroid. During the course of this study I have determined by the inoculation of considerable numbers of roaches (partial results in table 3) that rapidly growing bacteria will kill the insects quickly even though the original injection is very small, although a thousand times as many organisms of a slow-growing strain, such as the diphtheroids or some yeasts, will not kill. This points only to the fact that these cockroaches are able to destroy relatively small numbers of not too virule bacteria and is proof neither for nor against the identity of the diphtheroids.

Mercier (1907), after preliminary examination, assumed that the "normal" cockroach oothecae are free of contaminating microorganisms. Gropengiesser (1925), Glaser (1930), and Bode (1936) accepted this assumption and used it as evidence that the bacteria they cultivated from the oothecae were the "symbionts." These workers overlooked the possibility of frequent inclusion between the oothecal lips of normal saprophytic organisms from the vagina or oviducts of the cockroach. Although this incidence of infection is not high (20 to 25 per cent in the animals used in these experiments), it is frequent enough that it cannot be overlooked. Conceivably in some stocks many more females could carry such infection of a nature harmless to the roach but very disconcerting to meddlesome bacteriologists. The high correlation in frequency and kind of organisms cultivated from entire oothecal contents and from the lips of the ootheca indicate that most of these infections came from the vagina, were at first limited to the region beyond the tips of the eggs (figure 3), and occasionally spread into the space around the eggs. Less than half as many contaminations were encountered following the described technique when fresh oothecae were used as the source of inoculum than when embryos of over ten days' development were used. There was, however, no appreciable difference in frequency of positive growths between new and old ootheca when the entire capsule was macerated. Techniques in which the oothecae are macerated or are opened by separation of the lips get the advantage of all possible oothecal infections. This probably accounts for most of the positive cultures of roach "symbionts" from oothecae.

TABLE 3

Injection of roaches with bacteria

ORGANISM	NUMBER OF ORGANISMS INJECTED	EXPERIMENTAL ANIMALS	ANIMALS SURVIVING 3 DAYS
Serratia marcescens	100,000	5	0
	50,000	5	1
Pseudomonas fluorescens	400,000	5	0
Tetragenous sp.	1,500,000	10	5
Sarcina sp.	3,000,000	4	4
Diphtheroid I	2,000,000	10	5
Diphtheroid II	5,000,000	4	4
	15,000,000	8	2

Various bacteria isolated from the roaches themselves, grown on agar slants 24 to 48 hours, and suspended in Belar's solution, were injected into last instar *P. americana* through the fovea of the femur in quantities of 0.05 to 0.15 ml. Numbers of bacteria in the suspension were determined by counting in a hemacytometer. Death from injected bacteria usually occurred within 20 to 60 hours, if at all, depending on virility and dose.

As for the other reported positive cultures of roach symbionts, little need be said. Mercier (1907) and Gropengiesser (1925) did not perfect their technique. Bode (1936) showed quite clearly that *Bacillus cuenoti* is not the roach symbiont by failing to cultivate anything from aseptically raised roaches, by failing with hanging drops, and by getting *B. cuenoti* only in liquid media with large amounts of inoculation material, in which case he greatly increased the chances of contamination and lost all chance of control or even of seeing what was happening. Yet, for some reason, not made clear in his report, Bode maintains, doubtfully, that *Bacillus cuenoti* is the roach "symbiont."

Mercier, Gropengiesser, and Bode did the thing that has been done too often in the cultivation of intracellular "symbionts": i.e., they depended on the accuracy of their technique and on the improbability of a constant contaminant as proof that the organism cultivated was the true "symbiont" (for other such examples see Schwartz, 1935) in spite of the fact that this organism (*Bacillus cuenoti*) is motile, sporeforming, stains solidly, and is strongly gram-positive, all of which characteristics contrast violently with the bacteroids in the cockroaches. Certain bacteria are known to change some of their characteristics under changed conditions, but all such drastic changes as this should be seriously questioned before being accepted. Glaser (1930) relied not only on technique but on striking similarities (general form, barring, nonmotility, and nonsporulating properties) between the cultivated organism and the roach symbionts, and in addition attempted serological comparisons. Hoover (1945), possibly, has hit upon one of the factors that has been greatly responsible for repeated failures in cultivation; i.e., time. Whether or not she has actually cultivated the symbiont of *Cryptocercus* is not at all certain from her report. From the results of the series of experiments described above, it is probable that all of these cultures are contaminants, and not the bacteroids.

Failure to cultivate the bacteroids of the roach may be due to any one of the following factors or a combination of these, and should in no way be interpreted to mean that these bacteroids are not living units:

(1) These bacteroids may be so highly specialized for intracellular existence that they will not grow in any other medium. Many parasites, even of higher types, have not yet been grown outside their chosen habitats. These bacteroids are possibly more like rickettsiae than like bacteria, or, as Wallin (1925) suggested, perhaps the "symbiont" has become part of the host. Certainly they are adapted to a very specific set of conditions, and they cannot reasonably be expected to grow under conditions that do not closely approximate their normal habitat in most fundamentals.

(2) The proper medium may not yet have been developed. We know very little definitely of the conditions in which these bacteria live such as osmotic pressure, specific salt, protein, and fat concentration; hydrogen ion concentration; and other conditions existing within the cell. There are many other bacteria and rickettsiae known, both saprophytic and pathogenic, that require special media. Some common pathogens have only recently been cultured by using new developments in media and by utilizing the living medium of chick embryos. It is yet possible that the proper medium may be developed either by trial and error or by careful analysis and duplication of the normal bacteroid habitat.

(3) These bacteroids may have a normal reproductive cycle that is too slow for our culture methods. Other work now in preparation for publication shows that during the stage of most rapid increase of bacteroid numbers (late embryo) it takes 10 days to double the number. Unless this reproductive rate can be drastically increased, cultivation of the bacteroids by ordinary bacteriological methods is unlikely. Schwartz (1924) and Glaser (1930) hypothesized a chemical control of the host over its "symbiont" numbers, and they think that

this controlling element must be diluted before the "symbionts" can be cultivated. Contrary to their belief, I have found the "symbiont" increase to follow quite closely the weight increase of the host, and no factor controlling the bacteroid numbers in the cockroaches other than the regular factors controlling cell division and growth in the host could be detected. Theoretically, it is possible to adapt the "symbionts" to artificial media that would be so much more favorable for their development that they would make a really appreciable growth in a month or two.

There is no evidence to support the idea advanced by Mercier (1907) and reiterated by Gropengiesser (1925) that a yeast may at times displace the bacteroids. At times, however, the bacteroids in poorly fixed and insufficiently stained sections have somewhat the appearance of yeast because of swelling and vacuolization (figure 4). As Buchner (1930), Fraenkel (1921), and Bode (1936) have failed to find a yeast in the roach tissues, it is possible Mercier and Gropengiesser misinterpreted poor material. Sarcinae occurred frequently in the cultures in this series, as in those of Mercier (1907) and Gropengiesser (1925). These, as well as the yeasts, are probably saprophytes or temporary parasites that at times live in the vagina or oviduct and from there may be enclosed within the ootheca. There is, at present, no basis for hypothesizing that these organisms play a role as secondary "symbionts."

Neither has anyone produced any evidence to support the contention (Buchner, 1930) that the bacteroids of cockroaches may be different organisms in different localities or under different conditions. Extensive study of many species of cockroaches and of the same species from widely separated localities (Kansas, Indiana, Florida, and Massachusetts) forces the conclusion that the intracellular symbionts of the cockroaches are as specific and constant as are the roaches themselves. In this series of studies, there is no indication other than that each species of cockroach is the specific host for one organism, and that one type only may live within any one roach bacteriocyte. Roaches of all ages of several species (*Periplaneta americana*, *P. australasiae*, *Blatta orientalis*, *Blatella germanica*, *Parcoblatta pennsylvanica*, *P. uhleriana*, *P. virginiana*, *P. latta*, *Nyctobora noctivaga*, *Eurycotis floridana*, and *Crytocercus punctulatus*) were carefully studied for occurrence, behavior, morphology, and staining reactions of symbionts. Most of these species were used both fresh from the field and after long maintenance under laboratory conditions. Many individuals were subjected to drastic experimental conditions, such as partial and complete starvation; unbalanced diet; extreme temperature variations; injections of yeasts, bacteria, and chemicals into the hemocoele; X-ray and ultraviolet treatment; and deliberate neglect resulting in overcrowding and accumulation of their own wastes. In all cases, the morphological and staining characteristics of the symbionts remained remarkably constant, and indicate that the symbionts are at least all within the same genus, the differences being merely slight average variations in size and in arrangement of bars. Only extreme variations in the symbiont of any roach species can be distinguished from any other. *Parcoblatta* symbionts are a little thinner (0.8 μ as against 1 to 1.1 in other genera); *Blatta* symbionts have a greater tendency to form chains of three or four rods, and are more noticeably curved; *Blatella* symbionts are more uniform in length; and *Cryptocercus* symbionts have broader dark bands and fewer light-staining areas than are usual in the others. I have not found the extremely slender symbionts described by Hoover (1945). Thes variations are so slight that I cannot definitely identify any host by study of a film preparation of symbionts.

As to the taxonomic position of the cockroach symbionts, I have only to offer that they are generically all the same. Their morphology and staining reactions do not definitely place them in any established genus of bacteria. They are gram-positive but not strongly so. They are uniform rods with rounded ends, straight to half-moon curve, that vary in length from 1.5 to 6.5 μ in the same host. They divide by fission, typically into two unequal rods that tend to remain united until, or past, the next division time. They are not acid-fast, and form no spores even after the death of the host. They show a barred pattern with some stains, especially with the Giemsa stain and haematoxylin, and in some cases with carbolfuchsin and with Gram's stain, and to a much less extent with Alberts, Neisser's, and Loeffler's stains. No cilia have been demonstrated and no movement of the symbionts has been observed by me or recorded by others. These symbionts, then, have some of the characteristics of *Corynebacterium*, of *Spirillum*, and of *Bacterium*.

The barred appearance of intracellular "symbionts" following Giemsa stain or haematoxylin seems to be rather common as it has been specifically mentioned in the gut "symbiont" of *Rhodnius* (Wigglesworth; 1936); in the "symbiont" of the bedbug mycetocyte (Buchner, 1930); in the ant "symbiont" (Lilienstern, 1932); in the root nodule "symbionts" of legumes; and possibly in others that have escaped my attention. Of these, only the "symbiont" of *Rhodnius* has been designated by the investigator as a diphtheroid. Possibly this disposition of materials within the "symbiont" body is a characteristic derived from the intracellular existence rather than an indication of relationship to the *Corynebacterium*. It would probably be desirable for taxonomists so to define the *Rickettsiaceae* (as proposed by Steinhaus, 1940) as to include most of the bacteria-like intracellular symbionts, at least until they can be demonstrated to belong to the *Bacteriaceae*.

Glaser's species of diphtheroids (*Corynebacterium periplaneta*) is probably a valid species, if we understand that it has not been demonsrated to be the intracellular symbiont of *Periplaneta*.

SUMMARY AND CONCLUSIONS

Various techniques for manipulations of intracellular bacteroids, and all kinds of media on which growth of the intracellular bacteroids of cockroaches have been reported, were tried.

Numerous attempts to cultivate the intracellular bacteroids of *Periplaneta americana*, *Blatta orientalis*, *Parcoblatta pennsylvanica*, and *Cryptocercus punctulatus* failed.

All bacteria suspected of being the bacteroids were definitely eliminated by reducing their frequency of occurrence with each refinement in technique, and by cultivating their counterparts from other sources.

Hanging drop cultures, tissue cultures, implants on chick chorioallantois, and injections of the bacteroids into chick amniotic cavities—all failed to produce any perceptible increase in bacteroid numbers.

It is improbable that yeasts or cocci ever displace the normal bacteroids of the cockroach.

It is very doubtful that anyone has yet cultivated the intracellular bacteroids of any cockroaches.

The intracellular bacteroids of the blattids studied are generically the same and should probably be included in the *Rickettsiaceae*.

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SHIGELLA TYPES ENCOUNTERED IN THE MEDITERRANEAN AREA[1]

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The present communication is to report the occurrence of dysentery microorganisms in the Mediterranean area. Similar data on the *Salmonella* types found in that vicinity are given by Bruner and Joyce (1946).

Between September, 1943, and October, 1945, more than 1,344 *Shigella* cultures were examined serologically and biochemically. These were isolated in the Section of Bacteriology of the 15th Medical General Laboratory during food-handler examinations and in the investigation of outbreaks of bacillary dysentery or were submitted for identification from the laboratories of U. S. Army hospital units or medical laboratories. Of the 1,344 cultures, 1,182 were recovered in Italy between January, 1944, and October, 1945. Most of the remainder were isolated in North Africa (Casablanca to Bizerte) before January, 1944, although a few cultures originated in Sicily and southern France.

METHODS

Shigella cultures were identified tentatively by slide agglutinative tests with absorbed antiserums and confirmed by agglutination in serially diluted absorbed antiserums or by one-tube tests (Ewing, 1944). The serological methods employed were based largely on the work of Boyd (1938, 1940) but were similar to those described by other investigators (Wheeler, 1944*a*, 1944*b*; Weil *et al.*, 1944).

All cultures were confirmed as members of the genus *Shigella* by their biochemical reactions. This included tests for the utilization of glucose, lactose, sucrose, mannitol, salicin, and citrate; for indole and hydrogen sulfide production; for urea hydrolysis; and for motility. This list was considered the minimum that could be used. Mannitol was included, not because of any value in the establishment of a culture as a member of the genus *Shigella*, but because of its differential value within the group.

More extensive biochemical studies were made when the need was indicated. Many microorganisms were encountered that proved to be different, serologically or biochemically, from the described shigellae. Some of these were studied extensively (Wheeler *et al.*, 1946; Ewing, 1946), and others are being investigated at present.

RESULTS AND DISCUSSION

Table 1 lists all of the *Shigella* types encountered in the period mentioned. All duplicate cultures were eliminated, and each tabulation represents a case or a carrier. The figures for *Shigella paradysenteriae*, Flexner III (Z), and *Shigella* species, Q 771, found in hospital patients, are somewhat weighted since 18 of the former and 13 of the latter were isolated from two small outbreaks of bacillary dysentery in Algeria. Likewise, 7 of the *Shigella paradysenteriae*, Boyd IV (P 274), cultures originated in a single outbreak in Italy. All others came from sporadic cases.

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TABLE 1

Shigella types encountered in the Mediterranean area

SHIGELLA	FOOD HANDLERS			HOSPITAL PATIENTS	MISC.	TOTAL
	Civilian	American	German			
S. paradysenteriae						
F. I (V)	3	0	4	19	1	27
F. II (W)	55	1	7	109	8	180
F. III (Z)	31	4	6	94	6	141
F. IV (B. 103)	31	0	2	26	1	60
F. V (B. P119)	7	1	1	30	2	41
F. VI (B. 88, Newcastle)	46	4	4	137	5	196
B. I (B. 170)	0	0	0	10	0	10
B. II (B. P288)	0	0	0	1	0	1
B. IV (B. P274)	12	3	0	46	0	61
B. V (B. P143)	0	0	0	1	0	1
Lavington (*S. etousae*)	12	0	1	14	2	29
Unclassified	21	1	1	24	6	53
S. alkalescens	11	4	4	40	3	62
S. 2-193 (2372)	6	0	1	0	2	9
S. dispar	40	2	0	22	3	67
S. dispar-related	10	2	0	14	1	27
S. sonnei	71	2	4	136	16	229
S. dysenteriae (Shiga)	1	0	0	4	1	6
S. ambigua (*schmitzii*)	18	1	1	22	2	44
Species, Q771	0	1	0	46	0	47
Species, Q1167	1	0	0	8	0	9
Species, Q1030	0	0	0	8	0	8
Species (*Shigella*-like)	10	2	0	7	0	19
Paracolon, related to *S. alkalescens*	3	0	0	8	1	12
Paracolon, related to Boyd IV	1	0	0	1	0	2
Paracolon, related to Q771	1	0	0	1	1	3
Total........................	391	28	36	828	61	1,344

F., Flexner; B., Boyd.

Except for a few Algerians, the civilian food handlers listed in the table were Italian. The majority of hospital patients were U. S. Army personnel; a few were U. S. Navy personnel, French Colonial soldiers, or German prisoners of war. The miscellaneous group was made up of cultures from individuals about whom little information was obtained.

Shigella sonnei occurred most frequently. Two hundred and twenty-nine or

17 per cent of the total number of cultures were of this type. *Shigella paradysenteriae*, Flexner VI (*Shigella newcastle*, Boyd 88), was second in order of frequency (14.6 per cent); *Shigella paradysenteriae*, Flexner II (W), third (13.4 per cent), and *Shigella paradysenteriae*, Flexner III (Z), fourth (10.5 per cent).

Subgrouping of the Flexner types was not done routinely. Examination of a group of *S. paradysenteriae*, Flexner II, cultures selected at random indicated that the majority were of the IIa variety (Wheeler, 1944*a*), but some were IIb, and a few appeared to be neither IIa nor IIb. *Shigella paradysenteriae*, Flexner I (V), occurred much less frequently than the other members of the Flexner group (2.0 per cent) and were, for the most part, of the I type rather than the I.III or VZ (Weil *et al.* 1944), although a few of the latter were isolated.

Four other microorganisms occurred commonly. They were *Shigella dispar* (5.0 per cent), *Shigella alkalescens* (4.6 per cent), *S. paradysenteriae*, Boyd IV (P 274; 4.5 per cent), and *Shigella paradysenteriae*, Flexner IV (Boyd 103; 4.4 per cent), in the order of their frequency of occurrence.

The microorganisms which were more prevalent in the civilian food-handler applicants tended to appear more commonly in cases of bacillary dysentery. *S. paradysenteriae*, Flexner IV, and *S. dispar* are apparent exceptions to this statement. The former type was more prevalent during 1945 than during 1944. Of 17 cultures isolated during 1944, only one was recovered from a civilian food-handler applicant (Italian), whereas 15 were from hospital patients; one culture was placed in the miscellaneous group for that year. During 1945, 30 cultures of Flexner IV were isolated from civilians and only 10 originated in hospital patients. *S. dispar* appeared in food handlers more often than in patients with dysentery. Serological varieties of this microorganism occurred in cases, however, from which no other bacterium of significance was recovered. The disease was of a mild nature in most cases and tended to become chronic in some instances. The same *S. dispar* serotype was isolated repeatedly from the feces of one individual for nearly a year. During this time the person suffered recurrent attacks of diarrhea accompanied by abdominal pains.

Although the shigellae of the Boyd group other than *S. paradysenteriae*, Boyd IV (P 274), were not common, they were isolated from cases in which they were undoubtedly the etiological agent.

The cultures listed in table 1 as *Shigella* species, 2-193, are similar to *S. alkalescens* in regard to their biochemical characteristics but differ antigenically. This type is related serologically to *Shigella paradysenteriae*, Boyd V (P 143), to certain other members of the Boyd group, to *S. dispar*, and to a lesser extent to *S. paradysenteriae*, IV, V, and Y (unpublished data; Wheeler *et al.*, 1946; Carpenter and Stuart, 1946). Further studies on this interesting serotype are in progress.

Shigella paradysenteriae, Lavington, is a "new" serotype found in North Africa and Italy (Ewing, 1946). It is identical to cultures isolated in England by Lavington *et al.* (1946) and by Heller and Wilson in France (1946).

The unclassified group, which consisted of about 4 per cent of the cultures, is made up of *Shigella paradysenteriae*-like isolates. Some are related serologically to the Flexner types, some to the Boyd types, and others bear little or no sero-relation to any described type with which they were compard. This group, along with some mannitol-negative cultures, is being studied further.

Only one culture of *Shigella dysenteriae* was isolated from an Italian civilian food handler during nearly two years in Italy. Four cultures were recovered from patients in Italy (3 American, 1 Brazilian), but in each instance the history indicated that the infection was acquired outside Italy. The sixth culture (table 1) was submitted to us indirectly from the American University at Beirut.

Forty-four cultures of *Shigella ambigua*, representing 3.3 per cent of the total, were examined. Of the other mannitol-negative shigellae (Wheeler and Stuart, 1946; MacLennan, 1945), *Shigella* species, Q 771, occurred most commonly (47 cultures or 3.5 per cent). *Shigella* species, Q 1167 and Q 1030, cultures were encountered but they were not numerous. Two cultures of *Shigella* species, Q 771, labeled "inagglutinable Shiga" were received indirectly from the American University, Beirut.

Other mannitol-negative microorganisms are listed in the table as *Shigella*-like cultures. A few of these were related to known types, but in most cases they were serologically distinct from described shigellae.

Several motile cultures which proved to be identical to Sachs B.81 and B.105 and similar to no. 29911 of Stuart *et al.* (1943) were studied but were not included in table 1. Other paracolon bacteria were studied and were included in the table because of their sero-relation to *Shigella* types. Some of these, related to *S. alkalescens* or to *S. paradysenteriae*, Boyd IV (P 274), have been reported (Wheeler *et al.*, 1946) Three motile paracolon bacteria antigenically related to *Shigella* species Q 771 have also been studied and reported (Wheeler and Stuart, 1946).

SUMMARY

A report is made on 1,344 *Shigella* and *Shigella*-like cultures isolated in the Mediterranean area. The microorganisms were typed by serological methods and confirmed as shigellae by their biochemical reactions.

A table is included which lists the various types and their sources. Of the total, 828 cultures were isolated from hospital patients, 455 from food-handler applicants, and 61 made up a miscellaneous group.

The eight types encountered most frequently were as follows, in the order of their occurrence: *Shigella sonnei; Shigella paradysenteriae*, Flexner VI, Flexner II, Flexner III; *Shigella dispar; Shigella alkalescens; Shigella paradysenteriae*, Boyd IV (P 274); and *Shigella paradysenteriae*, Flexner IV.

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COMPARATIVE STUDIES OF ENTEROCOCCI AND ESCHERICHIA COLI AS INDICES OF POLLUTION

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The presence of coliform bacteria in water and in or on other foods depends on many environmental factors, each of which must be determined separately for each item of food in question. When a correlation is found to exist between insanitary methods of food production and the presence of *Escherichia coli* in the finished product, the presence of confirmed fecal strains of coliform organisms can be interpreted as having sanitary significance. Under these circumstances, however, if environmental factors tend to permit or promote multiplication of the normal flora of organisms, together with the coliform contaminants, an interpretation of the sanitary significance of *E. coli* becomes involved. Despite these limitations, the coliform bacteria have long been considered "the only bacterial group which can be used with even a reasonable degree of accuracy as a measure of pollution" (Hunter, 1939). It is not improbable that intestinal microorganisms, other than *E. coli*, have not been investigated because of technical difficulties. With the development of "SF" medium (Hajna and Perry, 1943) and the application of a few taxonomic requirements (Sherman, 1937), the enterococci represent a group of intestinal microorganisms which can now be readily isolated and identified. The applicability of the enterococci as an index of pollution thus merits consideration.

It has been demonstrated that enterococci are normally present in the intestinal tract of man and many warm-blooded animals (Sherman, 1937; Ostrolenk and Hunter, 1946). The work of the latter demonstrated that, in 37 per cent of 51 fecal specimens examined, enterococci occurred in equal or in greater numbers than *E. coli*. In the remaining 63 per cent of the specimens, *E. coli* exceeded enterococci numerically by from one to five decimal dilutions. The lower number of enterococci, as compared with *E. coli*, in human and animal feces does not in itself necessarily minimize the potential sanitary significance of fecal streptococci. It does, in fact, support the group as a more reliable index of pollution, provided there is a significant relationship between the presence of the organism and recognized pollution sources. Other factors that must be considered include distribution and reproductivity in nature, survival of temperature and chemical treatments, and longevity.

METHODS

Comparative studies were made of 53 specimens of commercial black walnut and pecan meats and fresh animal feces to determine the most efficient method and medium for the recovery of enterococci. Previous studies with "SF" broth (Ostrolenk and Hunter, 1946) revealed that approximately 7 per cent of the specimens examined gave false positive reactions. It was therefore anticipated that similiar results would be obtained on the examination of soils and washings from commercial foods. As a consequence, Hajna and Perry's "SF" broth was modified to contain 6.5 per cent sodium chloride, 0.4 g K_2HPO_4, and 0.15 g KH_2PO_4 per liter, and bromthymol blue indicator was substituted for bromcresol purple. This modified medium was evaluated against the original "SF" medium with incubation at 45.5 C, both in a constant temperature water bath and in a water-jacketed incubator. Preincubation at 37.5 C was also tried. Daily examinations of all inoculated tubes for 72 hours revealed that the method most productive of fecal streptococci consisted of the use of Hajna and Perry's unmodified "SF" broth for primary enrichment, incubated at 45.5 C in a water-jacketed incubator for 24 and 48 hours. Broth tubes showing acid production were streaked on "SF" agar ("SF" broth plus 1.5 per cent agar) and the plates were then incubated at 45.5 C for 24 or 48 hours. Five hundred and thirty-one colonies, which produced acid on "SF" agar plates and consisted of gram-positive streptococci, were isolated for study. These subcultures conformed to the taxonomic classification for enterococci (Sherman, 1937), i.e., possessing the ability to grow at 37.5 C in glucose broth containing 6.5 per cent sodium chloride, in alkaline glucose broth with a pH of 9.6, and in milk containing 0.1 per cent methylene blue, and to acidify and coagulate litmus milk. No culture produced typical hemolysis on Casman's blood agar (Casman, 1946) incubated in a candle jar at 37.5 C.

Following the development of the methods described above, data were accumulated on the longevity of enterococci and *E. coli* in soil, feces, and on foods. Studies were also made to determine the occurrence of these organisms on various commercial foods and their distribution in food-producing establishments. Parallel coliform determinations were made in the conventional manner using standard lactose broth followed by streaking on Levine's eosin methylene blue agar. Typical, well-isolated colonies were fished for confirmation in "IMVIC" media.

EXPERIMENTAL

Survival studies. To determine the longevity of enterococci as compared with *E. coli* under varying conditions of storage and from various sources, the following survival studies were conducted:

Fresh rat and mouse feces were stored in 140-mm open petri dishes at room temperature and at 7.22 C (45 F). Virgin soil and soil fertilized with chicken manure were each thoroughly mixed and then stored in open 500-ml glass beakers at room temperature and at 7.2 C. Five pounds each of pecan meats and soil were artificially contaminated with 18-hour washed suspensions of *E. coli* and enterococci isolated from rat feces. The pecan meats and soil were then stored in large, flat, shallow, open pans at room temperature.

Fifty-gram amounts of each of the soil and pecan specimens and 5-gram amounts of each of the fecal specimens were examined periodically for the test organisms. The soil fertilized with chicken manure ("B" in table 1) contained initially more than 100 enterococci but less than 1,000 per gram, and *E. coli* was demonstrated in a maximum of 1/1,000-gram portion of the soil washings. Eleven examinations of this specimen, covering a period of 160 days, revealed no fecal streptococci after 21 days in the portion stored at room temperature ("B"), but *E. coli* was present in 10-gram amounts of soil on the 66th day. In the second portion of the same soil ("B-1"), stored at 7.2 C, both test organisms were recovered on the 109th day but not on the 123rd day. In the unfertilized soil, stored at room temperature ("A"), no *E. coli* could be demonstrated after the initial examination prior to storage whereas enterococci were present in 1-gram amounts on the 109th day. In the refrigerated specimen ("A-1"), *E. coli*

TABLE 1

Longevity of enterococci and Escherichia coli in soil, in animal feces, and on food

MATERIAL	ORGANISM	NUMBER OF DAYS IN STORAGE										
		0	7	21	28	40	66	84	109	123	130	160
Soil "A"	Strep.	10^{-1}	10^{-2}	10^{-1}	10^{-2}	10 g	10^{-1}	1 g	1 g	0	0	0
	E. coli	10 g	0	0	0	0	0	0	0	0	0	0
Soil "A-1"	Strep.		10^{-2}	10^{-1}	10^{-2}	10^{-1}	10^{-2}	10^{-2}	10^{-1}	10^{-2}	0	0
	E. coli		0	1 g	1 g	0	10 g	0	0	0	0	0
Soil "B"	Strep.	10^{-2}	10 g	10^{-1}	0	0	0	0	0	0	0	0
	E. coli	10^{-3}	10^{-3}	0	0	10 g	10 g	0	0	0	0	0
Soil "B-1"	Strep.		10 g	10 g	0	10^{-1}	10 g	1 g	1 g	0	0	0
	E. coli		0	1 g	0	10^{-1}	0	10 g	10^{-1}	0	0	0
Soil "C"	Strep.	10^{-5}	10^{-3}	10^{-5}	10^{-4}	10^{-4}	10^{-2}	10^{-3}	10^{-3}	10^{-3}		10^{-2}
	E. coli	10^{-7}	10^{-6}	10^{-6}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-4}	10^{-3}		10^{-2}
Pecans "P"	Strep.	10^{-4}	10^{-5}	10^{-4}	10^{-3}	10^{-3}	10^{-1}	10^{-1}	1 g	1 g		10^{-1}
	E. coli	10^{-7}	10^{-6}	10^{-7}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-7}		10^{-6}
Rat feces "R"	Strep.	10^{-6}	10^{-1}	0^{-3}	10^{-3}		0		1 g		10^{-2}	10^{-1}
	E. coli	10^{-5}	10^{-5}	0^{-1}	10^{-3}		10^{-2}		10^{-3}		0	0
Rat feces "R-1"	Strep.		10^{-3}	10^{-5}	10^{-4}		10^{-4}		10^{-1}		10^{-1}	10^{-2}
	E. coli		10^{-3}	10^{-5}	10^{-5}		10^{-5}		10^{-5}		10^{-4}	10^{-4}
Mouse feces "M"	Strep.	10^{-7}	10^{-6}	10^{-6}	10^{-6}		10^{-5}		10^{-5}		10^{-4}	10^{-2}
	E. coli	10^{-5}	10^{-5}	10^{-3}	10^{-2}		10^{-5}		0		0	0
Mouse feces "M-1"	"Strep.		10^{-6}	10^{-7}	10^{-5}		10^{-6}		10^{-6}		10^{-5}	10^{-3}
	E. coli		10^{-3}	10^{-7}	10^{-5}		10^{-3}		10^{-4}		10^{-2}	10^{-3}

Figures (10, 1, 10^{-1}, etc.) represent dilution (per g) at which organism was found; 0 means not found in 10 g.

survived for 66 days and enterococci for 123 days. Soil ("C"), which was artificially contaminated, showed a gradual reduction in numbers of both organisms from an initial count of approximately 10,000,000 per gram to 100 per gram following room storage for 160 days. The pecan meats ("P"), simultaneously contaminated with *E. coli* and enterococci, showed no apparent reduction in coliform count following 160 days' storage at room temperature. Under the same conditions of storage, the fecal streptococci practically disappeared during this period.

The results of the examination of rat and mouse feces stored at room temperature and at 7.2 C ("R", "R-1", "M", and "M-1") indicate that enterococci survive longer than *E. coli* under such conditions of storage, and that refrigeration has a preserving action on both test organisms (table 1).

Commercial foods. Three hundred and eighteen specimens, representing four classes of fresh and frozen foods, were examined for enteroccocci and *E. coli:* 150 domestically shelled nut meat samples (three varieties); 27 specimens of frozen fruits and berries representing seven varieties; 46 frozen vegetable samples (eight varieties); and 96 specimens of fresh-picked crabmeat representing claw meat, white meat, backfin lump, and fancy pack. The nut meat and fresh crabmeat specimens were examined shortly after commercial packaging, whereas the specimens of frozen fruits, berries, and vegetables were examined following storage of these products of from several months to more than one year. Neither coliform bacteria nor enterococci were isolated from 16 per cent of the nut meat specimens and 25 per cent of the crabmeat specimens; 7.3 per cent of the former specimens and 9.5 per cent of the latter specimens contained approximately the same number of *E. coli* and enterococci, whereas 53.3 per cent of the nut meat and 58.3 per cent of the crabmeat specimens contained enterococci without any evidence of *E. coli*. Both types of food showed 2 per cent of the specimens to contain *E. coli* but no demonstrable enterococci. In 21.3 per cent of the nut

TABLE 2

Incidence of enterococci and Escherichia coli on commercial foods

SUBDIVISIONS SHOWING	TYPES OF FRESH AND FROZEN FOODS							
	Nut meats		Fruits and berries		Frozen vegetables		Fresh crabmeat	
	(No.)	(%)	(No.)	(%)	(No.)	(%)	(No.)	(%)
No strep.; no *E. coli*	24	16.0	22	84.6	6	13.0	24	25.0
Strep. equal to *E. coli*	11	7.3	0	0	1	2.2	9	9.5
Strep.; no *E. coli*	80	53.3	3	11.5	38	82.6	56	58.3
E. coli; no strep	3	2.0	1	3.9	0	0	2	2.0
Strep. greater than *E. coli*	24	16.0	0	0	1	.22	4	4.2
E. coli greater than strep	8	5.3	0	0	0	0	1	1.0
Totals	150		26		46		96	

meat specimens and 5.2 per cent of the crabmeat specimens both fecal streptococci and *E. coli* were found, the enterococci occurring in larger numbers. Total bacterial counts were also obtained on all the specimens examined. The estimates of microorganisms ranged from less than 10,000 to greater than 20 million per gram. Fifty-six of the nut meat samples that contained enterococci and coliform bacteria but no *E. coli* had total bacterial counts of 300,000 or more per gram of specimen.

TABLE 3

Incidence of Escherichia coli and enterococci on surfaces in crabmeat plants

CULTURE FROM	CRABMEAT ESTABLISHMENT NUMBER													
	1		2		3		4		5		6		7	
	E.c.	FS	*E.c.*	FS	*E.c.*	FS	*E.c.*	FS	*E.c.*	FS	*E.c.*	FS	*E.c.*	FS
Pickers' hands	+	+	−	+	+	+	+	+	−	−	+	+	−	−
Picking knife	+	+	+	+	+	+	+	+	−	−	−	+	−	−
Picking table	+	+	+	+	+	+	−	+	−	−	+	+	−	−
Crabmeat									−	−	+	+	−	−
Pickers' hands	+	+	+	+	+	+	+	+	+	−	−	+	−	−
Picking knife	−	−	+	−	+	+	+	+	+	−	+	+	−	−
Picking table	−	−	+	+	+	+	+	+	+	+	−	+	−	−
Crabmeat									+	+	−	+	−	−
Pickers' hands	+	+	+	+	+	+	+	−	+	−	−	−	−	−
Picking knife	−	−	−	−	+	+	+	+	−	−	+	+	−	−
Picking table	−	−	+	+	+	+	+	+	−	+	−	−	−	−
Crabmeat									−	−	−	−	−	−
Pickers' hands			+	+	+	+	−	−			−	−	−	−
Picking knife			−	−	+	+	+	+			+	+	−	−
Picking table			−	−	+	+	+	+			−	−	−	−
Crabmeat											−	−	−	−
Pickers' hands							+	+			−	−		
Picking knife							−	+			+	+		
Picking table							+	+			−	−		
Crabmeat											−	+		
Packers' hands			+	+	+	+	−	−						
Packing table	−	−			+	+	+	+						
Crab shovel			+	+			+	+	−	+	−	+	−	−
Cooling floor			+	+	+	+	+	+	−	−	+	+	−	−
Crab bucket	−	−									+	+		
Misc. surfaces	+	+	−	−										
Total samples	12		16		15		19		14		23		18	

Designations: *E.c.* (*E. coli*); FS (enterococci); + (positive); − (negative).

Of the 26 specimens of frozen fruits and berries examined, 3 (11.5 per cent) contained enterococci but no *E. coli;* 1 (3.9 per cent) contained *E. coli* but no fecal streptococci. The remaining 22 specimens (84.6 per cent) contained neither enterococci nor coliform bacteria. Thirty-eight (82.6 per cent) of the frozen vegetables contained enterococci alone; 2 (4.4 per cent) contained fecal streptococci and *E. coli;* and the remaining 6 (13 per cent) contained neither organism (table 2).

Food establishment specimens. Seven crabmeat-producing establishments were studied for the presence of *E. coli* and enterococci. The following specimens were collected for examination: washings from the hands of the operators, swabbings from representative surfaces of the equipment used in picking and packing crabmeat, wash water, hand and crabmeat can chlorine dip waters, the cooling ice employed in the manufacturing process, and picked crabmeat. Of 117 specimens examined by the methods described, 45 (38.4 per cent) contained neither *E. coli* nor enterococci; 12 (10.3 per cent) contained no *E. coli* but did contain enterococci; 4 (3.5 per cent) contained *E. coli* but no fecal streptococci; and 56 (47.8 per cent) contained both types of microorganisms (tables 3 and 4).

TABLE 4

Relative incidence of Escherichia coli and enterococci on surfaces in crabmeat plants

SPECIMENS SHOWING	NUMBER OF SPECIMENS
Escherichia coli negative; enterococci negative	45 (38.4%)
Escherichia coli negative; enterococci positive	12 (10.3%)
Escherichia coli positive; enterococci negative	4 (3.5%)
Escherichia coli positive; enterococci positive	56 (47.8%)
Total number of specimens examined	117
Number of specimens polluted (index enterococci)	68 (58.1%)
Number of specimens polluted (index *E. coli*)	60 (51.3%)

It should be noted (table 3) that in the food establishments numbered "3" and "7" an excellent correlation between sanitary conditions and the presence of enterococci and *E. coli* was obtained. Although both establishments possessed essentially all the equipment necessary to produce a wholesome, unpolluted product, rodent infestation, poor supervision, and lack of personal hygiene in plant number "3" resulted in the pollution of all surface items examined. In plant "7", good control over rodent contamination, proper supervision of operations, and the control of personal hygiene among the employees resulted in the elimination of both *E. coli* and enterococci in the specimens examined. The correlation between plant sanitation and the presence of intestinal types of bacteria on contact surfaces is further reflected in the data shown in table 4. Sixty specimens (51.3 per cent) were found to be polluted as judged by the presence of *E. coli*, whereas 68 specimens (58.1 per cent) were polluted when enterococci were used as the index of pollution.

SUMMARY

A method is described for the isolation of fecal streptococci, employing incubation at 45.5 C in 0.05 per cent sodium azide broth ("SF" broth) as a primary enrichment, followed by streaking on the same medium solidified by 1.5 per cent agar ("SF" agar), and incubating the plates at 45.5 C.

By this method, 531 cultures were studied (from nut meats, frozen fruits and berries, frozen vegetables, and freshly packed crabmeat) and found to be predominantly *Streptococcus faecalis* or *Streptococcus liquefaciens*.

Enterococci in artifically contaminated soils and pecan meats and in normal feces appear to survive longer than *Escherichia coli* under identical conditions of storage.

The adverse effect of high total bacterial counts on the isolation of *Escherichia coli* did not obtain with the use of "SF" media to isolate enterococci. It is suggested that this observation enhances the value of fecal streptococci as an index of pollution.

Data obtained from food-producing establishments show excellent correlation to exist between sanitation and recovery of both *Echerichia coli* and enterococci.

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STUDIES ON SOME BIOLOGICAL ASPECTS OF DIHYDROSTREPTOMYCIN

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The catalytic conversion of streptomycin to a dihydro compound was recently described and some of the biological characteristics of the latter were noted (Peck *et al.*, 1946; Fried and Wintersteiner, 1947; Bartz *et al.*, 1946). Peck and his coworkers (1946) reported that dihydrostreptomycin showed *in vitro* activity against *Bacillus subtilis*, and Bartz *et al.* (1946) also reported activity against *B. subtilis* as well as against some ten other species. *In vivo* studies by Peck *et al.* (1946) showed that approximately twice as much dihydrostreptomycin trihydrochloride as streptomycin trihydrochloride, calcium chloride double salt was required to protect 50 per cent of the mice against one lethal dose of *Salmonella schottmülleri*.

Dihydrostreptomycin is not inactivated by cysteine, hydroxylamine (Peck *et al.*, 1946), semicarbazide (Fried and Wintersteiner, 1947; Bartz *et al.*, 1946), nor by treatment with mild alkali (Peck *et al.*, 1946; Fried and Wintersteiner, 1947; Bartz *et al.*, 1946). Hence, it is relatively stable compared to streptomycin.

The present paper deals with some comparative biological characteristics of streptomycin and dihydrostreptomycin. For the bacterial spectrum shown in table 1, streptomycin trihydrochloride prepared from the crystalline trihelianthate was used. The dihydrostreptomycin employed was made from this streptomycin preparation (Fried and Wintersteiner, 1947). In the remaining comparisons shown here (tables 2 and 3, and figure 1) a streptomycin sulfate preparation having an activity of ca. 400 units per mg and its corresponding dihydro compound were used.[1]

IN VITRO ACTIVITY OF DIHYDROSTREPTOMYCIN IN COMPARISON WITH THAT OF STREPTOMYCIN AGAINST VARIOUS ORGANISMS

In table 1 are shown the minimal inhibiting concentrations (M.I.C.) of pure dihydrostreptomycin trihydrochloride as compared with pure streptomycin trihydrochloride for 10 species or strains of bacteria. The concentrations, which were determined in 0.8 per cent tryptone broth[2] at pH 7.5, are given in terms of weight rather than in terms of units so that comparisons between the two compounds may be made directly instead of comparing each with a standard strep-

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[1] We wish to thank Drs. Fried and Wintersteiner of the Division of Organic Chemistry, The Squibb Institute for Medical Research, for supplying the streptomycin and dihydrostreptomycin used throughout this work.

[2] The *in vitro* tests with the two species of mycobacteria were made in a modified Kirschner's medium.

tomycin preparation. Since the molecular weights of the two compounds are so similar (691 for streptomycin trihydrochloride and 693 for dihydrostreptomycin trihydrochloride) direct comparison of activities in terms of weight is permissible. Each figure in the table represents an average of 10 or more assays.

It will be noted that the organisms are listed in order of decreasing relative sensitivity to dihydrostreptomycin. Thus, although the dihydro compound is 13 per cent more active against *Mycobacterium smegmatis* than is streptomycin, its activity approximately equals that of streptomycin against *Aerobacter aerogenes*, *Bacillus subtilis* (Merck), *Klebsiella pneumoniae*, and *Serratia marcescens*. Toward one strain of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Mycobacterium tuberculosis*, and *Bacillus* sp., strain 290, dihydrostreptomycin shows decreasing activity in that order, relative to streptomycin; showing only one-third the inhibitory action of the parent substance against *Bacillus* sp. 290.

TABLE 1

Comparative in vitro activities of streptomycin and dihydrostreptomycin against various organisms

TEST ORGANISM	M.I.C.		ACTIVITY RATIO
	Streptomycin trihydrochloride	Dihydrostreptomycin trihydrochloride	
	µg/ml	*µg/ml*	
Mycobacterium smegmatis	1.54	1.36	1.13
Aerobacter aerogenes	0.086	0.082	1.05
Bacillus subtilis (Merck)	0.069	0.068	1.01
Klebsiella pneumoniae (ATCC 9997)	0.080	0.081	0.99
Serratia marcescens	0.084	0.089	0.94
Staphylococcus aureus (P-209)	0.046	0.055	0.84
Pseudomonas aeruginosa	0.56	0.82	0.68
Mycobacterium tuberculosis H37Rv*	3.36	5.66	0.59
Bacillus subtilis (558)	0.092	1.60	0.57
Bacillus sp. (290)	0.012	0.036	0.33

* The authors wish to express their appreciation to Miss Clara McKee for conducting the *in vitro* tests with *Mycobacterium tuberculosis*.

RESPONSE OF STREPTOMYCIN-RESISTANT ORGANISMS TO DIHYDROSTREPTOMYCIN

A strain of *Hemophilus influenzae*, type B, grown resistant to streptomycin (Alexander *et al.*, 1946) and an unidentified gram-negative rod described by Smith[3] (1946) as being very resistant to this antibiotic were tested as to sensitivity to streptomycin and to the dihydro compound. In Levinthal's broth 25,000 units of streptomycin per ml failed to inhibit the *Hemophilus* culture, and in yeast beef broth 2,500 units per ml failed for the unidentified rod. Equivalent amounts of dihydrostreptomycin were similarly ineffective.

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[3] This culture was obtained from Miss Dorothy Smith and Dr. Hans Molitor, of the Merck Institute for Therapeutic Research, to whom the authors are indebted.

EFFECT OF CULTURE MEDIUM MODIFICATIONS ON IN VITRO ACTIVITY OF DIHYDROSTREPTOMYCIN

It has previously been shown that raising the pH of test media increases the antibiotic activity of streptomycin (Waksman and Schatz, 1945), whereas increasing the tryptone concentration in a test broth decreases the activity of this antibiotic for *K. pneumoniae* (Donovick and Rake, 1946). The results of a comparative study of the effects of pH and tryptone concentration on the activity of dihydrostreptomycin and its parent substance are shown in tables 2 and 3. The procedure for determining the minimal inhibiting concentrations was the same as that used in earlier work with streptomycin (Donovick and Rake, 1946).

TABLE 2

Comparison of effect of pH on in vitro activity of streptomycin and dihydrostreptomycin against K. pneumoniae

pH OF TEST BROTH*	M.I.C.	
	Streptomycin sulfate	Dihydrostreptomycin sulfate
	μg/ml†	*μg/ml*†
6.4	0.39	0.39
7.2	0.16	0.13
7.8	0.14	0.10
8.4	0.072	0.057
9.0	0.063	0.036

* Test broth consisted of 0.8 per cent tryptone in water and was adjusted with HCl or NaOH to the pH indicated.

† The concentration is given in actual weight and *not* in terms of free streptomycin base. Comparisons are possible because the dihydrostreptomycin used was prepared directly from the streptomycin employed here, the only difference being that the former had been reduced by hydrogenation. The streptomycin had an activity of ca. 400 units per mg.

TABLE 3

Effect of tryptone concentration on in vitro activity of dihydrostreptomycin against K. pneumoniae at pH 7.0 to 7.1

CONCENTRATION OF TRYPTONE IN TEST BROTH	Streptomycin	Dihydrostreptomycin
	μg/ml	*μg/ml*
0.5	0.076	0.055
0.75	0.124	0.108
1.00	0.168	0.166
2.00	0.380	0.256

* See footnotes to table 2.

It will be noted that the *in vitro* activity of both streptomycin and dihydrostreptomycin increase with a rise in pH and decrease with a rise in tryptone concentration. Under some circumstances, such as at pH 9.0 and in some concentrations of tryptone broth, the *in vitro* activity of dihydrostreptomycin against *K. pneumoniae* appears to exceed slightly that of streptomycin. In general, however, the activities of the two substances under these changing conditions are very similar.

EXCRETION OF DIHYDROSTREPTOMYCIN IN MICE

In the upper section of figure 1 is shown the rate of recovery of dihydrostreptomycin in the urine of mice following a single, subcutaneous dose equivalent to 3,450 units of streptomycin per mouse (172,500 units per kilo). For purposes of comparison, data from earlier work on the excretion of streptomycin in mice (Rake and Donovick, 1947) are included in the figure. The procedure employed in the present work was similar to that used in the previous study, and in both cases the data are based entirely on repeated bioassays of the urine samples.

The over-all recovery in the current experiment was ca. 41 per cent of the dose administered, which, as shown in figure 1, is somewhat lower than our findings with streptomycin in mice, but which is within the broad range of experimental variation involved in such work.

In order to compare the rate of excretion of dihydrostreptomycin with that of streptomycin it is convenient to plot the data, taking the total amount of antibiotic recovered in the urine in each experiment as 100 per cent. When this is done (*vide* lower section of figure 1), the streptomycin curves from three experiments are found to coincide very closely and that of dihydrostreptomycin shows a slightly slower rate of excretion. Thus, while 34 to 44 per cent of the recovered streptomycin appeared in the urine during the first half-hour, in the case of dihydrostreptomycin only 20 per cent of the antibiotic recovered was found during this period. Further, whereas 80 to 88 per cent of the streptomycin recovered appeared during the first hour, it required 2 hours to reach 88 per cent in the case of dihydrostreptomycin. However, excretion of a drug is a complex function of blood levels, which in turn are determined by absorption and distribution, as well as by renal activity. Consequently, it would be unwise for the present to carry conclusions any further than to say that in mice the dihydro compound is excreted in a qualitatively similar fashion to streptomycin.

TOXICITY OF DIHYDROSTREPTOMYCIN

Streptomycin having an *in vitro* activity of ca. 400 units per mg and its corresponding dihydro preparation were tested in cats[4] for blood-pressure-lowering effects, and in mice for immediate and delayed toxicity when administered intravenously. The cat test, as described by the Food and Drug Administration in *Minimum Specifications for Streptomycin*, is based upon the comparison of the

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[4] The authors are indebted to Dr. J. C. Burke, of the Pharmacological Development Department, E. R. Squibb and Sons, for conducting the cat tests.

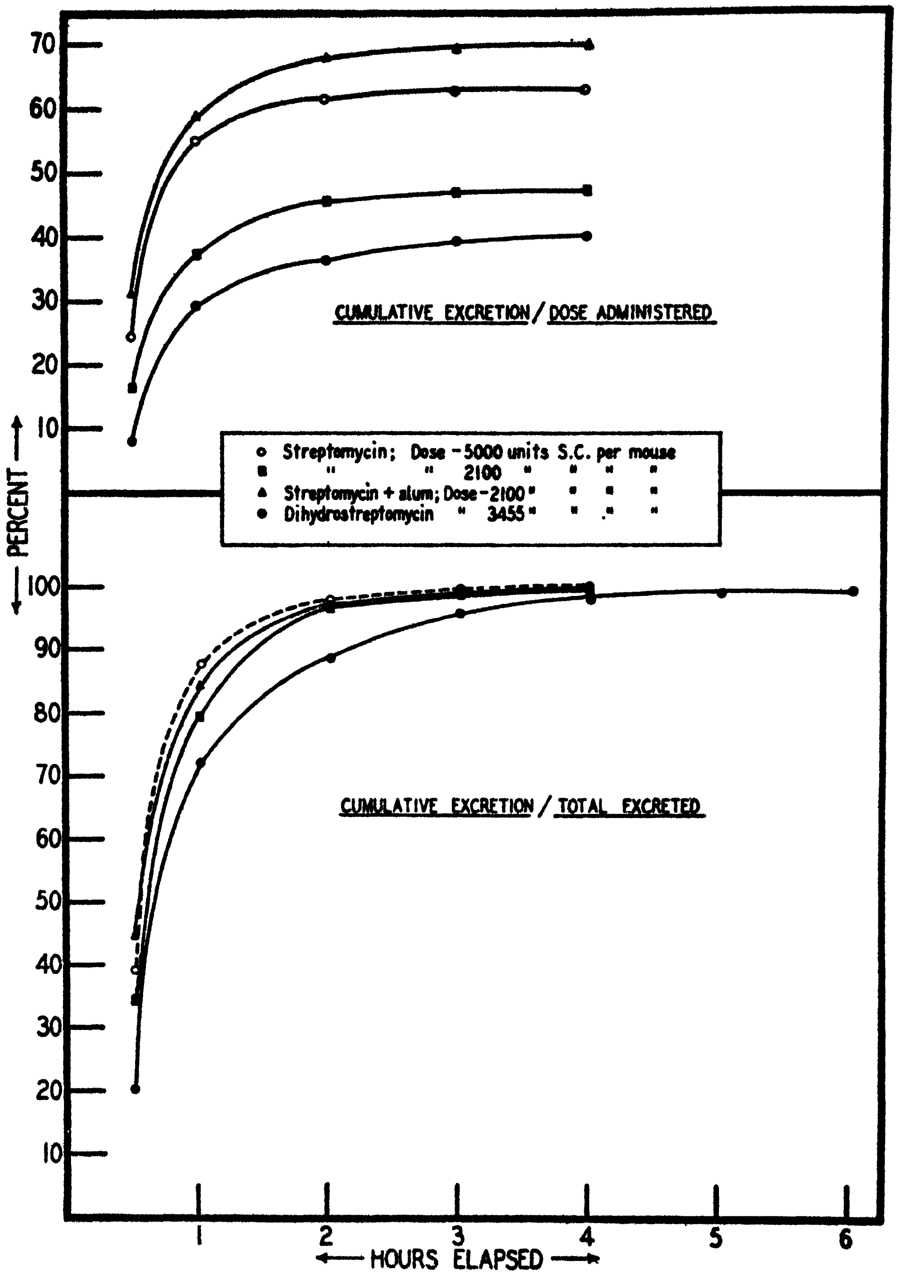

Fig. 1. Excretion of Streptomycin and Dihydrostreptomycin in Mice

blood-pressure-lowering effect of a given dose of streptomycin with that of a standard dose of histamine, under fixed conditions. The histaminelike action of some impure streptomycin preparations is due to impurities.

The streptomycin used in these studies caused a blood-pressure-lowering effect of >6 × the standard dose of histamine, but after reduction with hydrogen, the resultant dihydrostreptomycin preparation caused an effect in this test approximately equal to the standard histamine dose. Should dihydrostreptomycin prove to be a therapeutic agent approximately equal to streptomycin, the process of conversion of streptomycin to dihydrostreptomycin by hydrogenation will have a secondary value of reducing some impurities capable of this histaminelike action.

The intravenous toxicities of the two compounds in mice were not essentially different from each other. The LD_{50} of the streptomycin used was between 4.0 and 4.5 mg per mouse (equivalent to ca. 1,600 to 1,800 units per mouse or 91,500 to 103,000 units per kilo), whereas that of the reduced preparation lay between 5.0 and 5.5 mg per mouse. Shock began to appear in mice at doses of 2.5 mg of streptomycin and 3.0 mg of dihydrostreptomycin. However, the differences may again be referred to impurities. All deaths occurred within 1 hour (usually in considerably less time) from the time of injection, and mouse weight and food intake data gathered during a 21-day period following the injection showed no abnormalities in either group. Autopsies at the end of this period on mice that received 4.5 mg of streptomycin and 5.0 mg of dihydrostreptomycin revealed no pathological changes in either group.

DISCUSSION

Dihydrostreptomycin is a unique derivative of streptomycin in that the antibiotic activity against many organisms remains largely intact in spite of the reduction of the aldehyde group in streptomycin to a carbinol group. When this aldehyde group is treated with carbonyl reagents (Brink *et al.*, 1945; Donovick, Rake, and Fried, 1946) or when oxidized to a carboxyl group (Fried and Wintersteiner, 1947), the antibacterial activity is lost. The question then arises whether the biological activity of dihydrostreptomycin is an inherent characteristic of the compound per se or whether a conversion to streptomycin occurs before antibiotic action is possible.

Studies attempting to solve this interesting problem are underway. One approach is through the formation of maltol from streptomycin by treatment with mild alkali (Schenck and Spielman, 1945) and the failure of this treatment to produce maltol from dihydrostreptomycin (Peck *et al.*, 1946; Fried and Wintersteiner, 1947). Maltol can be detected by its characteristic ultraviolet absorption (Fried, Coy, and Donovick, unpublished). In one preliminary study we found that no maltol could be demonstrated by this procedure[5] when *K. pneumoniae* cells were prepared in a very heavy suspension in a dihydrostreptomycin solution, the mixture was incubated for 24 hours at 37 C, the cells were removed by centrifugation, and the supernatant fluid was examined for maltol.

In the investigation of the excretion of dihydrostreptomycin in mice, described above, aside from *in vitro* assays, the urine was also examined by the ultraviolet absorption procedure. Further work is needed, however, as the results were ambiguous. After the mice had been injected there appeared in the urine substances other than maltol that absorbed ultraviolet light at the wave length used for detecting maltol. The very high concentrations of antibiotic in the ½-hour and 1-hour samples, representing 70 per cent of the total activity recovered, makes it possible to say that little or no maltol was present in these fractions. In later samples, which contained smaller amounts of antibiotic, the interfering substances were of such magnitude, relative to the concentration of antibiotic, as to nullify quantitative interpretation.

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[5] We wish to thank Dr. J. Fried and Mr. F. Russo-Alesi for making the ultraviolet absorption studies.

SUMMARY

Dihydrostreptomycin, which is an antibiotic compound derived from streptomycin by reduction with hydrogen, has *in vitro* activity equal to that of streptomycin for some bacterial species, but for others its activity is only a fraction that of streptomycin. In the case of one strain of *Mycobacterium smegmatis* its activity is slightly greater than that of streptomycin.

Changes in pH and tryptone concentration in test broths affect the antibacterial activities of streptomycin and the dihydro compound similarly.

Two species of bacteria resistant to streptomycin were also shown to be resistant to dihydrostreptomycin.

The excretion rates of these two compounds as well as their toxicities in mice are very similar.

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PRACTICAL MEDIA AND CONTROL MEASURES FOR PRODUCING HIGHLY TOXIC CULTURES OF CLOSTRIDIUM BOTULINUM, TYPE A[1]

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The wide assortment of preserved foods from which botulism has been contracted plainly indicates that *Clostridium botulinum* can utilize a variety of naturally occurring nutrients for production of toxin. Yet in the laboratory, meat infusion, peptone media are customarily employed for this purpose. When detailed studies on the purification and characterization of the toxin were contemplated, the expense of infusion media and the difficulty of obtaining meat during wartime presented serious difficulties. A search was, therefore, undertaken for media that could be prepared in quantity at minimum cost and yet provide the necessary nutrients for the production of highly toxic cultures of *Clostridium botulinum*, type A. A study was also made of certain biochemical changes that accompany toxin production, in order to provide, if possible, relatively simple and rapid methods for determining when to harvest cultures containing maximum levels of toxin. The results of this investigation, as reported herein, have provided means for obtaining sufficient toxin to permit crystallization and biochemical study of the pure toxin by Lamanna *et al.* (1946). A similar investigation of media for the preparation of toxoid was conducted concurrently by Nigg *et al.* (1946), who adopted somewhat different formulae to avoid the introduction of allergenic substances that might interfere with clinical use.

To a considerable degree the development of practical media was advanced by the unpublished contributions of a number of independent investigators whose names are listed in the acknowledgment. In addition to the unpublished data cognizance was taken of certain pertinent observations recorded in the literature, such as the work of Burke (1919), Elberg and Meyer (1939), Gladstone and Fildes (1940), Dack and Wood (1928*a*; 1928*b*), Dack, Wood, and Dehler (1928), Wagner, Meyer, and Dozier (1925), Dozier, Wagner, and Meyer (1924), Stark, Sherman, and Stark (1928), and Lamanna and Lewis (1946). The reviews on bacterial nutrition by Knight (1938) and Peterson and Peterson (1945) were also helpful.

METHODS

The "Hall" strain of *C. botulinum*, type A, obtained through the courtesy of Dr. J. H. Mueller, Harvard University, was selected for this investigation because unpublished work by McCoy and Sarles (1943) indicated that it produced more toxin per unit of culture than any other strain tested by them.

Inocula for experimental work were prepared by first transferring approximately 0.1 ml of the turbid supernatant fluid from a well-stirred meat mash stock culture to a tube or bottle containing a medium similar to those being tested. After incubation at 34 C for 18 to 30 hours appropriate aliquots were transferred aseptically to experimental media. Initially inocula were sometimes taken directly from meat mash cultures to test media; however, this introduced the possibility of carrying over unknown ingredients which would influence the results. Final data were obtained with inocula from cultures which had undergone several serial transfers in appropriate liquid media at 24- to 48-hour intervals.

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[1] Studies conducted at Camp Detrick, Frederick, Md., from June to December, 1943.

The corn steep liquor employed in many experiments was obtained in wooden barrels from the Corn Products Refining Company, Argo, Illinois. As received, it contained approximately 50 to 60 per cent solids. When media containing this material were sterilized in the autoclave, massive precipitates were encountered. To avoid this difficulty the corn steep liquor was diluted sufficiently with water to make a thin, free-flowing slurry, which was then adjusted to pH 8.4 to 9.0 with concentrated sodium hydroxide. The mixture was heated to boiling and clarified by centrifugation or filtration. Approximately 40 per cent of the solids were removed. The filtrate could then be incorporated in culture media with the formation of relatively little precipitate upon sterilization. The quantities of corn steep liquor used in this investigation have been recorded in terms of the approximate amounts of total solids.

When casein or powdered milk were to be used, 10 per cent suspensions were first prepared by adding each 10-g lot of powder to 90-ml portions of tap water, adjusting to pH 7.6 to 8.0 with N/1 NaOH, and stirring in a Waring "blendor" until finely dispersed, stable suspensions were obtained. When necessary, the total volumes were brought to 100 ml by further additions of water. Appropriate aliquots of the 10 per cent suspensions were then mixed with other ingredients to give the desired final concentration of casein or milk.

Test media were usually prepared in 100- to 150-ml amounts in 6- or 8-oz screw-capped prescription bottles. The hydrogen ion concentration was adjusted with sodium hydroxide or hydrochloric acid so that the media were at pH 6.8 to 7.4 after sterilization in the autoclave at 120 C for 15 to 20 minutes. The bottles of media were inoculated soon after preparation with relatively heavy inocula (usually 2 to 5 per cent by volume) and were incubated at 34 C. Under these conditions no special procedures were required to insure adequate anaerobiosis, provided agitation was minimized to avoid undue aeration of the medium.

Growth was estimated by turbidity, evolution of gas, proteolysis, and microscopic observation of films stained by Gram's method.

For the determination of toxicity, cultures were first diluted in a solution of the following composition:

Gelatin (Difco)	2	g
$NaH_2PO_4 \cdot H_2O$	7.25	g
Na_2HPO_4	3.70	g
Distilled water to make	1,000	ml
Approximate pH	6.5	

White mice weighing 18 to 24 g were then injected intraperitoneally with 0.5-ml aliquots of each dilution. Because of the limited number of animals available, only two to four mice were injected with each dilution, and statistically significant LD_{50} values could not be obtained. For this reason the data have been recorded in terms of an approximate MLD based on the greatest dilution causing death of half or more of the mice within 4 days.

Determinations of pH were made with a standardized glass electrode apparatus. Chemical analyses were performed according to the procedures given by Hawk and Bergeim (1942). Tests for reducing substances and nonprotein nitrogen were made, respectively, by the Folin-Wu and Koch-McMeekin methods.

RESULTS

Selection of Ingredients for Production of Toxic Cultures

Preliminary comparison of casein, gluten, and peptone. The initial trials were directed toward a preliminary evaluation of different protein sources to replace the casein digest and meat peptone commonly employed in media for *C. botulinum*, type A. A basal medium containing yeast extract, cerelose (commercial glucose), thioglycolic acid, and tap water was used to compare casein, corn gluten, Difco peptone, and pepticase (casein digest). When inoculated lightly, the various culture media listed in table 1 showed appreciable differences in toxin production. The basal medium without proteinaceous material or with 1 per cent Difco peptone failed to support growth. Media containing either casein or corn gluten in concentrations of 2.5 per cent allowed production of only 1,000 MLD of toxin per ml, whereas the pepticase medium supported the production of 100,000 MLD per ml.

When mixtures were employed, 2 per cent casein plus 0.5 per cent pepticase permitted the development of 1,000,000 MLD per ml. As the pepticase was decreased below this figure and the casein increased proportionally, toxin production was correspondingly reduced. Mixtures of casein and peptone allowed production of even less toxin than the casein alone. Because of physical difficulties of handling corn gluten in liquid media, less extensive trials were made with this material than with the casein, but it appeared from a single trial, using a combination of 0.1 per cent pepticase and 2.5 per cent gluten, that this mixture was equivalent to a similar medium in which casein replaced the gluten, for both produced 10,000 MLD of toxin per ml.

Since some toxin had been produced from the casein alone, it seemed possible that by increasing the inoculum or adjusting environmental conditions this protein might completely substitute for pepticase. A second series of experiments, was, therefore, conducted to compare casein digest with casein. In this case both the commercial product, pepticase, and a laboratory pancreatic digest of casein were employed in the basal medium mentioned above. After 3 days' incubation at 34 C, all three media contained approximately 100,000 MLD of toxin per ml. It seemed probable from these data that casein was as useful as casein digest for toxin production, thus eliminating the laborious digestive processes.

To determine the least amount of casein that would be practicable, varying concentrations from 0.05 to 2 per cent were tested. The results given in table 2 indicate that 0.25 to 0.5 per cent casein was adequate in the yeast extract cerelose medium previously described. In this range, 100,000 to 1,000,000 MLD of toxin per ml could be obtained regularly, and no further increase in yield was observed when the casein content was increased to 1 or 2 per cent. Lesser amounts than 0.25 per cent failed to support adequate growth, thus making abundant toxin production impossible. For routine toxin production 0.3 per cent casein has been used successfully.

TABLE 1

Variations in toxin production with culture media containing different kinds and combinations of proteinaceous material

SUPPLEMENTS TO BASAL MEDIUM* (%)				MLD PER ML × 10^5
Peptone	Pepticase	Casein	Gluten	
0	0	0	0	0†
1	0	0	0	0†
1	0	1.5	0	<0.01‡
0.1	0	2.4	0	<0.01‡
0.01	0	2.5	0	<0.01‡
0	2.5	0	0	1.
0	1.0	1.5	0	1.
0	0.5	2.0	0	10.
0	0.1	2.4	0	0.1‡
0	0.01	2.5	0	0.01‡
0	0	2.5	0	0.01‡
0	0	0	2.5	0.01
0	0.1	0	2.5	0.1

* Basal medium: Difco yeast extract 0.5 per cent, glucose (cerelose) 0.6 per cent, thioglycolic acid 0.05 per cent, and tap water. Adjusted to pH 7.5. Dispensed in test tubes (10 ml per tube) and sterilized in the autoclave at 120 C for 15 minutes.

Supplements: Bacto peptone was obtained from Difco; pepticase (a tryptic digest of casein) received from Sheffield Farms, Inc.; technical grade of acid-precipitated casein was procured from Baker Chemical Company; gluten was a granular product made commercially from corn (manufacturer unknown).

Inoculum: One loopful of 5-day meat mash culture per tube.

Incubation: Three days at 34 C.

† No growth.

‡ Slight growth.

Supplements to replace yeast extract. To produce the quantities of toxin needed for biochemical studies, it was necessary to grow the cultures in large containers, such as 5-gallon carboys, and to have an adequate inoculum constantly available for successive production lots. Serial transfers of the culture in the production medium were therefore desired in order to provide a relatively large volume of inoculum at frequent intervals for the large culture vessels. Successive transfers in the casein, yeast extract, glucose medium resulted, however, in partial loss of toxicity after the second transfer and eventual failure of growth after 4 to 6 transfers. From these results it appeared that the medium was deficient in some constituent necessary for the continued development of *Clostridium botulinum.*

A search was then undertaken to find supplements which would either replace or fortify the yeast extract. For this purpose a basal medium composed of 2 per cent casein, 0.6 per cent cerelose, and 0.05 per cent thioglycolic acid in tap water was employed. Serial transfers were made at daily intervals, using 2 per cent by volume of the preceding culture. Table 3 records the results of the first and fourth transfers after each had been incubated for 3 days at 34 C. The first transfer showed growth and toxin production to the extent of 100,000 to 500,000 MLD per ml in all media. After the fourth transfer appreciable differences in growth and toxin production were evident, depending upon the supplement employed. The basal medium without supplements, as well as the casein digest medium used to prepare the initial inoculum, showed no growth. Supplementing the basal medium with barley malt extract or beef infusion permitted slight growth but little or no toxin production. Yeast extract, ground beef, and barley sprouts extract supported some growth through the fourth transfer but permitted production of toxin only to the extent of about 100,000 MLD per ml. Corn steep liquor, cerophyl, or a mixture of yeast extract and cerophyl allowed adequate growth and production of 500,000 to 1,000,000 MLD of toxin per ml. Among the more useful of these supplements corn steep liquor seemed to be the most economical and readily available. For this reason it was used in later experiments to replace the yeast extract.

TABLE 2

Concentration of casein required for toxin production

CASEIN	MLD PER ML × 10^5	
	A	B
(per cent)		
2	10	5
1	10	1
0.5	10	5
0.25	5	1
0.10	*	*
0.05	*	*

Basal medium: Difco yeast extract 0.5 per cent, glucose (cerelose) 0.6 per cent, thioglycolic acid 0.05 per cent, and tap water. Adjusted to pH 7.4. Sterilized in the autoclave at 120 C for 15 minutes.

Inoculum: Two per cent of a 24-hour culture in basal medium plus 2.5 per cent pepticase.

Incubation: Three days at 34 C.

Casein: Technical grade procured from Baker Chemical Company.

A and B: Indicate replicate trials.

* Growth slight or absent; no toxin formation detected.

Elimination of thioglycolic acid. Up to this point, thioglycolic acid had been incorporated into the medium as a reducing agent to aid the initiation of growth, but there was no proof that it contributed beneficially to the medium. Comparison of casein, corn steep liquor, glucose media with and without 0.05 per cent thioglycolic acid showed, in fact, that abundant growth and toxin production to the extent of 500,000 MLD per ml could be obtained either with or without this reducing substance when 2 per cent of an actively growing culture was used as the inoculum. Although this constituent was periodically included in other test media, it was eventually abandoned because toxin production was not influenced by its absence.

TABLE 3

Influence of supplements on toxin production in casein, glucose, thioglycolate medium

SUPPLEMENTS	MLD PER ML × 10^5 AFTER SERIAL TRANSFERS INDICATED	
	1st	4th
(per cent)		
None	1	<1*
Yeast extract (Difco) 0.5	5	1
Corn steep liquor (clarified) 0.4 (solids)	5	5
Cerophyl† 0.5	1	5
Cerophyl 0.05	1	1*
Cerophyl 0.25 + yeast extract 0.25	5	10
Ground lean beef 0.5 (wet weight)	1	1*
Beef infusion 2.5 (based on wet weight of meat)	1	<1*
Barley malt extract 0.25 (based on weight of malt)	1	<1*
Barley sprouts extract 0.25 (based on weight of sprouts)	1	1
Casein digest‡ 2.5 (based on weight of casein)	5	<1*

Basal medium: Casein 2 per cent, glucose (cerelose) 0.6 per cent, thioglycolic acid 0.05 per cent, and tap water. Adjusted to pH 7.4. Sterilized in the autoclave at 120 C for 15 minutes. Readjusted to pH 6.8 to 7.2 with N/1 NaOH when necessary.

Inoculum: Media initially seeded with 2 per cent of a 24-hour culture containing penticase 2.5 per cent, Difco yeast extract 0.5 per cent, glucose 0.6 per cent, thioglycolic acid 0.05 per cent, and tap water.

Incubation: Three days at 34 C.

* Growth slight or absent.

† Dry, powdered, green vegetation commercially prepared.

‡ Formula as for inoculum above.

Selection of proteinaceous material. Using the corn steep liquor, cerelose basal medium, various protein materials were again compared with particular regard to their toxin-producing qualities. Table 4 shows that casein digest, casein from three different commercial sources, corn gluten, and powdered milk were equally satisfactory in that all of them permitted development of approximately 500,000 MLD of toxin per ml. Corn gluten was again regarded as undesirable because of its insolubility in liquid medium. Casein required careful manipulation to disperse it properly in the medium. Casein digest, though more easily handled, was available only in limited amounts and at high cost. Powdered milk had the advantage of ready availability, minimum processing requirements, and easy dispersion in the culture medium. For these reasons the medium composed of powdered milk, corn steep liquor, and glucose was selected for various additional tests to determine the minimum concentration of each ingredient required, the effect of supplements, and the possible influence of varying the amount of inoculum.

Although powdered milk was chosen for further experimental work there was no indication that the use of casein was fundamentally objectionable. Possibly elimination of the additional constituents of milk, especially lactose, would tend to discourage rapid growth and acid production by contaminants that occasionally find their way into mass cultures. In any event, either casein or powdered milk was as satisfactory as digests of casein or meat for the production of highly toxic cultures of the "Hall" strain, which is actively proteolytic and is, therefore, capable of decomposing protein as the culture develops.

TABLE 4

Equivalent toxin production in media containing milk, casein, or gluten

PROTEIN SOURCE	CONCENTRATION	MLD PER ML × 10^5	
		A	B
	(per cent)		
Casein digest (pepticase)	2.5	1	5
Casein (Baker)	2.0	5	5
Casein (Hercules)	2.0	5	5
Casein (Coleman & Bell)	2.0	5	5
Powdered skim milk	2.0	5	10
Corn gluten	2.0	5	5

Basal medium: Alkali-heat-treated corn steep liquor 0.4 per cent dry weight, glucose (cerelose) 0.6 per cent dry weight, thioglycolic acid 0.05 per cent by volume, and tap water. Adjusted to pH 7.5. Sterilized in autoclave at 120 C for 15 minutes. Adjusted after sterilization to pH 6.8 to 7.2 when necessary with N/1 NaOH.

Inoculum: Two per cent by volume of a 24-hour culture in basal medium plus 2 per cent casein.

Incubation: Three days at 34 C.

A and B: Indicate replicate trials.

Minimum effective concentrations of selected constituents. Table 5 summarizes the data concerning the minimum concentration of each ingredient required for maximum toxin production. All media permitted growth, but toxin production was maximum only when proper concentrations of all three constituents were used. This suggests that the requirements for growth and toxin production may not be identical in the case of *C. botulinum*. The omission of any one of the three ingredients prevented the accumulation of toxin in abundance, and when suboptimal amounts of various constituents were employed, toxin production decreased accordingly. The following are the approximate minimum concentrations which allowed maximum toxin production: 2 per cent powdered skim milk, 0.2 per cent corn steep liquor (total solids), and 0.3 per cent glucose. Inasmuch as the levels did not seem to be critical in the range of excess quantities, the amounts of glucose and corn steep liquor were increased to 0.5 and 0.4 per cent, respectively, for the routine production of toxin.

TABLE 5

Minimum effective concentrations of milk, glucose, and corn steep liquor for toxin production

CONSTITUENTS OF MEDIUM (PER CENT DRY WEIGHT)			MLD PER ML × 10^5
Milk*	Glucose†	Corn Steep‡	
6.0	0.5	0.4	5
2.0	0.5	0.4	5
0.5	0.5	0.4	<1
0.2	0.5	0.4	<1
0.05	0.5	0.4	<1
2.0	0.6	0.4	10
2.0	0.5	0.4	10
2.0	0.4	0.4	10
2.0	0.3	0.4	5
2.0	0.2	0.4	5
2.0	0.1	0.4	1
2.0	0	0.4	<1
2.0	0.3	0.8	5
2.0	0.3	0.4	10
2.0	0.3	0.2	10
2.0	0.3	0.1	5
2.0	0.3	0	<1
2.0	0	0	<1

Inoculum. Five per cent of a 24-hour culture prepared in the first medium listed in the table.

Incubation: Three days at 34 C.

* Powdered skim milk.

† Commercial quality sold under trade name, Cerelose. Sterilized separately and added aseptically to other constituents for this test.

‡ Heavy, fermented corn steep liquor was clarified and then used in amounts required to provide the total solids indicated.

Observations on added ingredients. It was well established, both from the literature and from experience gained during this investigation, that the toxin was relatively unstable when cultures were incubated for prolonged periods. One of the factors influencing the stability of the toxin is the change in hydrogen ion concentration resulting from the accumulation of metabolic products of the organisms. In the hope of finding materials which would tend to stabilize the toxin and allow greater accumulation in the culture medium, a variety of supplements were added to the milk, glucose, corn steep liquor medium described above. After inoculation, cultures were incubated at 34 C, and aliquots were removed on the first, second, and ninth days of incubation. Determinations of pH and toxicity were made on each sample, and the results are shown in table 6.

TABLE 6

*Influence of supplements on production and retention of toxin by C. botulinum (type A) in a milk, glucose, corn steep liquor medium**

SUPPLEMENT†	DAYS	pH	MLD PER ML × 10^5
Lard stick	1	5.9	>5
	2	6.0	10
	9	6.3	5
Peanut meal	1	5.9	5
	2	6.0	10
	9	6.3	1
Black peat	1	6.0	>5
	2	6.2	25
	9	6.8	<1
Blackstrap molasses	1	5.8	5
	2	5.9	25
	9	6.2	5
Cerophyl	1	5.8	5
	2	5.9	25
	9	6.2	5
Distillers' solubles	1	5.8	>5
	2	5.8	10
	9	6.2	5
None	1	5.8	5
	2	5.9	10
	9	6.2	5

Inoculum: Three per cent of a 24-hour milk, corn steep, glucose culture.
Incubation: 34 C.

* Basal medium: Dried skim milk 2 per cent, alkali-heat-treated corn steep liquor 0.3 per cent (dry weight), technical glucose 0.3 per cent, and tap water. Adjusted to pH 7.6. Sterilized at 120 C for 15 minutes.

† Supplements suspended in water, sterilized at 120 C for 15 minutes, and added aseptically to basal medium to give a final concentration of 0.2 per cent.

During the first 24 hours a marked drop from pH 7.0 to pH 5.8 to 6.0 occurred, and, in some cases at least, relatively large amounts of toxin accumulated. During the following 24 hours the pH remained unchanged or reverted slightly toward the alkaline side, and toxin production appeared to have reached the maximum level in most cultures. The basal medium contained 1,000,000 MLD of toxin per ml, and those cultures containing supplements had toxicities of the same order of magnitude, although the presence of cerophyl, blackstrap molasses, or black peat tended to give slightly higher values. By the ninth day of incubation the pH of most media had reverted to 6.2 to 6.3, although the ones containing black peat had risen to pH 6.8. The toxicity of all cultures had decreased until 500,000 MLD per ml or less remained. In the case of black peat, which showed the marked rise in pH, less than 100,000 MLD of toxin remained. These results indicate that the supplements tested were of no special value for enhancing the stability of toxin or markedly increasing the yield. In fact, it appeared that additional constituents might make the medium less desirable for toxin production because they added inert material, and in one case at least, the final pH of the culture was so altered that the toxin disappeared more rapidly than in the basal medium.

TABLE 7

*Influence of agitation and amount of inoculum on toxin production in milk, glucose, corn steep liquor medium**

AGITATION†	INOCULUM‡	FINAL pH§	MLD PER ML × 10^5 AT DAYS INDICATED		
			1	2	3
	per cent				
None	10	6.5	1	10	10
None	5	6.7	1	10	5
None	2	6.2	1	10	10
None	1	5.9	—	—	10
None	0.5	6.0	—	—	10
None	0.1	5.9	—	—	5
4–6 times	10	6.5	<1	<1	<1
4–6 times	1	6.7	—	—	<1
4–6 times	0.1	5.9	—	—	10

* Powdered milk 2 per cent, alkaline, heat-treated, filtered corn steep liquor 0.4 per cent, and glucose (cerelose) 0.5 per cent, mixed with tap water and adjusted to pH 7.4. Medium sterilized in the autoclave at 120 C for 15 minutes and then readjusted to pH 7.0 when necessary.

† Manual shaking, as commonly used for mixing cultures with dilution blanks, was applied during each working day but not at night.

‡ Sixth serial transfer in the foregoing medium was used after 24 hours of incubation.

§ Determined with glass electrode apparatus after 3 days of incubation at 34 C.

Influence of Inoculum and Agitation on Toxin Production

The fact having been established that the milk, glucose, corn steep liquor medium provided nearly optimum conditions for toxin production, a brief study was then made of the influence of agitation and of varying amounts of inoculum upon the rapidity of formation and final yield of toxin. Different lots of the medium were inoculated with 10, 5, 2, 1, 0.5, and 0.1 per cent of a 24-hour culture representing the sixth serial transfer in the same medium. These were incubated in a quiescent state at 34 C for 3 days. Duplicate cultures, which received 10, 1, and 0.1 per cent inocula, respectively, were agitated four to six times during each working day by shaking in a manner similar to that used for mixing cultures in dilution blanks. At night no shaking occurred. These were also incubated for 3 days at 34 C. Gross observations of the cultures indicated that those receiving 2 to 10 per cent inocula grew most rapidly, as judged by evolution of gas and proteolysis. These cultures were tested for toxicity at intervals of 1, 2, and 3 days after inoculation, but the remaining cultures were tested only on the third day. The results are shown in table 7.

Although the heavier inocula showed maximum toxin production in 2 days, they yielded no greater concentration of toxin at 3 days than the smaller inocula so long as the cultures were not disturbed during incubation. Even with 10 per cent inoculum, daily agitation prevented the accumulation of large amounts of toxin but did not inhibit growth. Only in the case of the agitated culture which received 0.1 per cent inoculum did sufficient toxin accumulate to be detected at the dilutions employed for animal injection. This culture was slow to initiate growth, and the period of rapid toxin accumulation apparently occurred during the night when the culture was not being disturbed. Thus agitation was not applied at the time when it would do most harm. From these results it would appear that even mild agitation, which permits entrance of air into the culture during the critical period of toxin accumulation, is harmful. On the other hand, the exact amount of inoculum is not critical in quiescent cultures, though some saving in time of incubation may be gained by using 2 per cent or more inoculum.

Biochemical Changes Associated with Toxin Production

Experience with the milk, glucose, corn steep liquor medium showed that subtle differences between batches of medium altered the rapidity of appearance, final yield, and stability of the toxin produced in the culture. Minor variations in the purity of the ingredients, method of preparation, quality of inoculum, and degree of anaerobiosis may have accounted for some of these unpredictable differences. Whatever the causes, a study of the biochemical changes accompanying toxin production in the culture seemed necessary in order to develop a method for predicting the time of maximum toxin accumulation. Direct measurement of toxicity in mice, requiring 2 or more days, was too slow for routine testing of cultures because marked deterioration of the toxin often occurred before the tests were completed.

In two series of experiments, the relationships of pH, nonprotein nitrogen, and reducing substances to toxicity were studied to learn how these factors varied in different media and whether biochemical tests could be used to follow toxin development.

Influence of corn steep liquor. The first series consisted of four lots of medium composed of 2 per cent powdered milk plus 0.3 per cent glucose, to three of which were added 0.8, 0.4, and 0.2 per cent corn steep liquor, respectively. The inoculum for each lot was 5 per cent by volume of a 24-hour culture grown in the foregoing medium containing 0.2 per cent corn steep liquor. From each lot of medium, aliquots were removed for testing before incubation and after 12, 24,

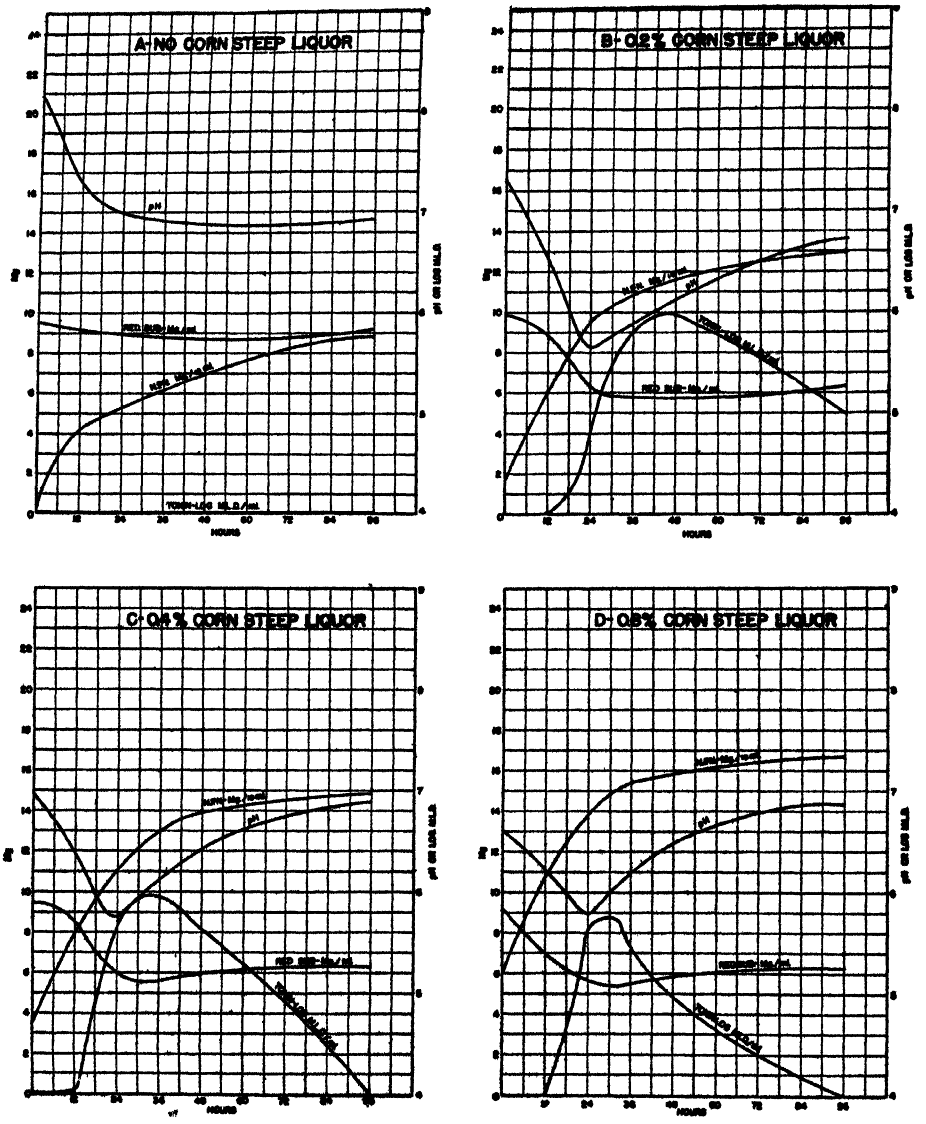

FIG. 1 CHANGES IN pH, NONPROTEIN NITROGEN (NPN), REDUCING SUBSTANCES (AS GLUCOSE), AND TOXICITY OF CLOSTRIDIUM BOTULINUM CULTURES IN MILK, GLUCOSE MEDIUM CONTAINING VARIOUS CONCENTRATIONS OF CORN STEEP LIQUOR

33, 45, 72, and 96 hours of incubation at 34 C. The values obtained for toxin, reducing substances, nonprotein nitrogen, and hydrogen ion concentration are presented in figure 1.

In the absence of corn steep liquor (figure 1A) no increase in toxin occurred during the entire 96 hours of incubation. The concentration of reducing substances showed only slight variation from the initial value. There was a tendency for the concentration to drop slowly and then to rise again to approximately the original level. The nonprotein nitrogen increased sharply from none to about 5 mg per 10 ml during the first 24 hours and then more slowly to give a final concentration of nearly 9 mg per 10 ml. The hydrogen ion concentration showed a rapid drop during the first 24 hours and thereafter leveled off at about pH 6.9, where it remained for the entire incubation period.

In the presence of corn steep liquor (figure 1B, C, and D) the behavior of the cultures was strikingly different. Between 12 and 24 hours a rapid rise in the toxin concentration occurred regardless of the amount of corn steep liquor in the various lots of medium. The maximum level was reached between 24 and 45 hours and thereafter declined gradually. The reducing substances declined rapidly from an initial level of 9 to 10 mg per ml to levels of approximately 5 to 6 mg per ml at about the interval when maximum toxin production occurred. The correlation of these two points was so marked that it would appear possible to determine the optimum time of harvesting toxic cultures by simply following at frequent intervals the concentration of reducing substances. After the low level of reducing substances was reached, a slight but consistent rise occurred during the later phase of incubation. The nonprotein nitrogen increased rapidly during the first 24 to 36 hours to different levels depending upon the amount of corn steep liquor present in the medium. Thereafter they rose more slowly. Hydrogen ion concentration increased sharply as demonstrated by the rapid drop from pH 7 to 5.6 to 5.8, which occurred during the first 24 hours. Thereafter the reaction gradually reverted toward the alkaline side, so that the final pH of all three cultures containing corn steep liquor was 6.7 to 6.9. The decline in toxin seemed to correspond roughly to this increase in alkalinity.

Influence of glucose. The second series of tests was conducted in a medium composed of 2 per cent powdered milk and 0.2 per cent corn steep liquor. To one portion of the medium no glucose was added, whereas the remaining three portions received 1.0, 0.6, and 0.3 per cent glucose, respectively. Each lot of medium received a 5 per cent inoculum as described for the preceding series. Aliquots were removed from all cultures at 6-hour intervals during the first 48 hours of incubation at 34 C, and at 12-hour intervals thereafter until 96 hours had passed. The same tests employed for the first series of trials, described above, were again performed on samples from the second series. The results are shown in figure 2.

In the absence of added glucose (figure 2A) a rapid but relatively slight rise in the toxin level occurred during the first 24 hours, which quickly dropped so that by 36 hours no more toxin remained than was initially added with the inoculum. Reducing substances first showed a slight and slow decrease, followed by a gradual rise to a level somewhat higher than the initial value of 4.1 mg per ml. Nonprotein nitrogen increased rapidly during the first 24 hours to about 9 mg per 10 ml and then leveled off at about 10 mg per 10 ml. The initial pH of the culture was 7.2 and the final pH was approximately the same. During the first

36 hours some drop in pH occurred to a minimum level of 6.7 which was followed by a period of reversion.

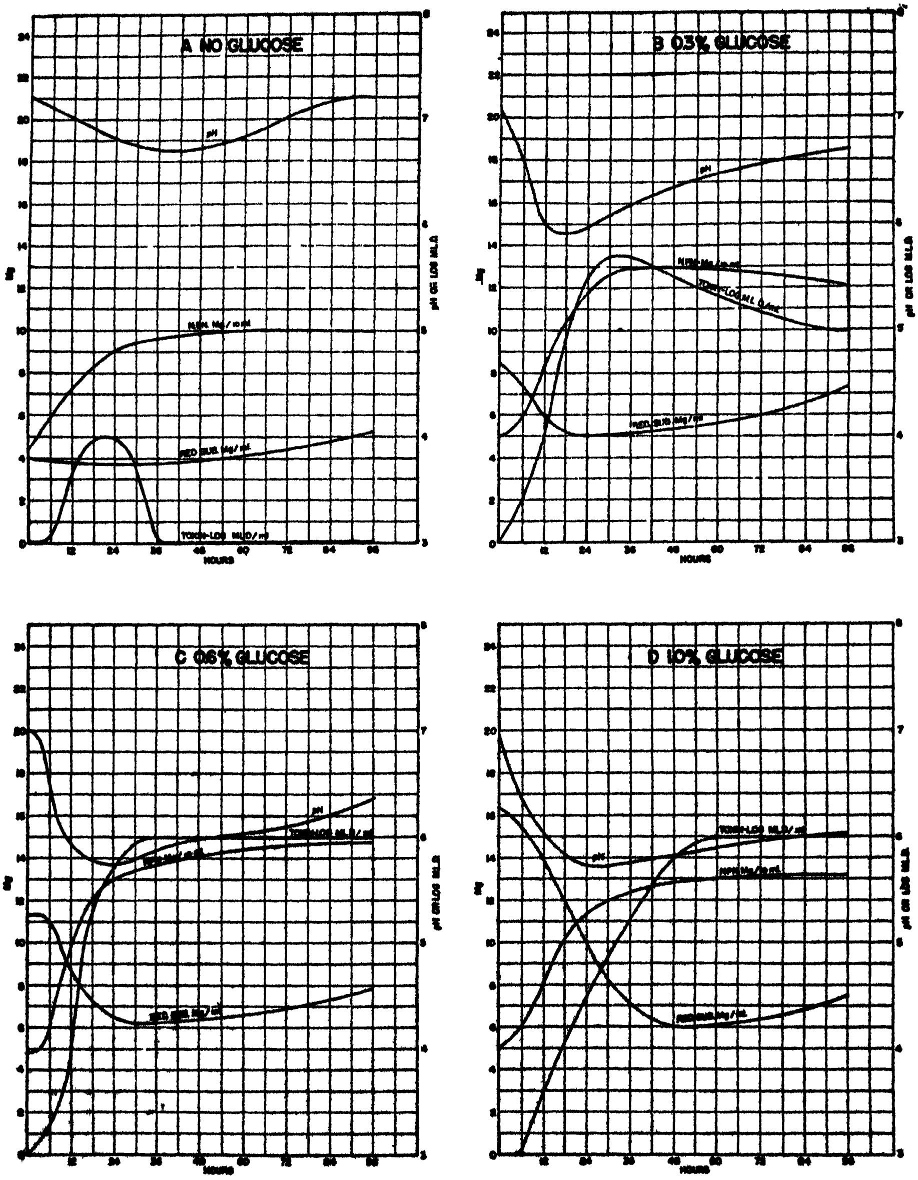

FIG. 2. CHANGES IN pH, NONPROTEIN NITROGEN (NPN), REDUCING SUBSTANCES (AS GLUCOSE), AND TOXICITY OF CLOSTRIDIUM BOTULINUM CULTURES IN MILK, CORN STEEP LIQUOR MEDIUM CONTAINING VARIOUS CONCENTRATIONS OF GLUCOSE

As the concentration of glucose in the medium was increased (figure 2B, C, and D) the rapidity of the toxin accumulation became slower, but in all instances reached a maximum level of 500,000 to 1,000,000 MLD per ml. Whereas the cultures containing 0.3 to 0.6 per cent glucose reached their maxima in approximately 30 hours, the culture containing 1 per cent glucose required nearly 60 hours. As in the preceding series of experiments, the reducing substances decreased rather sharply during the early phases of incubation from initial levels, which varied with the amount of added glucose, to low levels of 5.0 to 6.2 mg per ml at the time when maximum toxin production was reached. Here again the correlation between the minimum level of reducing substances and the maximum accumulation of toxin was so striking as to indicate the value of this chemical test for determining the optimum time for harvesting the toxin. The cause of the slow rise which characteristically occurred following the minimum level of reducing substances is unknown but presumably was related to the accumulation of protein degradation products, which possessed reducing characteristics. Nonprotein nitrogen increased rapidly from an initial level of 5 mg per 10 ml to approximately 13 mg per 10 ml during the first 24 to 36 hours of incubation and thereafter tended to level off. Although the most rapid increase in nonprotein nitrogen corresponded to the period of most marked toxin production, there was not the close correlation of critical values that has been noted in the case of reducing substances. In the presence of the various amounts of glucose a rapid drop in pH from approximately 7.0 to a minimum level of 5.7 to 5.9 occurred during the first 18 to 24 hours of incubation. In the presence of 0.3 per cent glucose a rather marked reversion to pH 6.7 occurred, but in the presence of 0.6 per cent glucose the final pH was approximately 6.4, and with 1 per cent glucose it rose only to about 6.0. Only in the first instance was there a marked decrease in toxin during the latter part of the incubation period, so that the stability of the toxin in the culture appeared to be closely related to the final pH.

Considering all the data presented in figures 1 and 2, it seemed that the rapidity of production, maximum level, and stability of the toxin were affected by the composition of the medium and the pH changes which occurred during incubation. The lack of corn steep liquor prevented full utilization of glucose, as indicated by the failure of reducing substances to decrease at the same rate or to the same extent as was observed in the presence of corn steep liquor. Likewise, the absence of glucose inhibited, to some extent, the utilization of proteinaceous materials, as indicated by the less extensive production of nonprotein nitrogen in this culture as compared to those with added glucose. In both cases toxin production was interfered with, yet growth occurred. When the medium contained all the ingredients in the proportions required for the production of large amounts of toxin, the characteristic changes in level of reducing substances were useful for determining when maximum concentration of toxin appeared in the culture. Although the nonprotein nitrogen was subject to characteristic alterations in level, it was not closely correlated with toxin production and was, therefore, of less value for following the progress of the culture.

It must be realized that the presence of contaminating organisms or the use of media not suitable to toxin production so alter the culture that the changes in pH and reducing substance no longer bear the same relationship to toxin production. Nevertheless, in pure cultures of *Clostridium botulinum* a considerable measure of control can be exercised to ensure recovery of maximum toxin by the determination of pH and reducing substances throughout the growth cycle.

RECOMMENDED MEDIA

Many variations of the casein (or powdered milk), glucose, corn steep liquor medium have been used successfully for production of the toxin of *C. botulinum* type A. The following two formulae are suggested because they are easily prepared and effective for routine use:

Formula I (to make one liter)

Solution A. Suspend 20 g powdered milk in 180 ml of water. Add N/1 NaOH slowly as required to disperse the milk, until a stable suspension has been obtained by shaking or stirring. If necessary add water to make 200 ml.

Solution B. Add 6 g commercial glucose (cerelose) to a volume of clarified corn steep liquor equivalent to 4 g total solids and dilute to 800 ml with water.

Thoroughly mix solutions A and B, adjust to pH 7.4 to 7.6, dispense into culture bottles, and sterilize in the autoclave for 15 to 20 minutes at 120 C. After sterilization the reaction of the medium should be pH 6.8 to 7.2.

Formula II (to make one liter)

Solution A. Add 3 g casein (technical grade) to 50 ml water previously alkalized with 0.5 g NaOH per liter. Adjust to pH 10.5 to 11.5 with 10 N NaOH. Mix thoroughly with a mechanical stirrer for about 30 minutes. Readjust pH if necessary and stir again until a stable suspension is obtained.

Solution B. Add 5 g commercial glucose (cerelose) to a volume of corn steep liquor equivalent to 5 g total solids and dilute to 800 ml with water.

Pour solution A into solution B and rinse the container with 100 ml of water to remove remaining casein. Adjust the mixture to pH 7.2 to 7.4, bring the total volume to 1,000 ml with water, dispense into culture bottles, and sterilize in the auotclave for 15 to 20 minutes at 120 C. After sterilization the reaction of the medium should be pH 6.8 to 7.2.

For best results the media should be freshly prepared, inoculated with 2 to 5 per cent by volume of an actively growing culture of *C. botulinum*, type A, and incubated at 34 C. If aeration of the culture is avoided by minimizing agitation and using culture vessels with a low exposed surface area per unit volume, 500,000 to 1,000,000 MLD of toxin per ml can be obtained regularly in 48 hours or less.

ACKNOWLEDGMENT

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SUMMARY

Practical liquid media, composed of readily available and relatively inexpensive ingredients, have been developed for the production of highly toxic cultures of the "Hall" strain of *Clostridium botulinum* (type A).

The peptones usually employed in culture media can be replaced by 0.25 to 0.5 per cent casein (technical grade) or 2 per cent powdered skim milk.

Clarified corn steep liquor (0.2 to 0.4 per cent total solids) is a more adequate supplement than yeast extract for toxin production in casein or milk media, as demonstrated by relative viabilities and toxicities after 4 to 6 serial transfers in media containing these materials.

The presence of available carbohydrate in the form of 0.2 to 0.6 per cent commercial glucose (cerelose) markedly increases the total yield of toxin and enhances its stability in the culture medium by retarding reversion of pH toward the alkaline side.

The milk, corn steep liquor, glucose medium yields 500,000 to 1,000,000 MLD of toxin per ml of culture when inoculated with 2 per cent of an actively growing culture of *C. botulinum* (type A, Hall strain) and incubated in a quiescent state at 34 C for 24 to 48 hours. Small inocula or mild agitation tend to retard toxin production, and the latter may greatly inhibit its accumulation.

Characteristic changes in pH, concentration of reducing substances, and levels of nonprotein nitrogen occur during incubation of the culture. The relationships of pH and reducing substances to rapidity of accumulation and stability of toxin provide valuable control measures for securing the maximum yield of active toxin.

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THE RELATION OF NATURAL VARIATION IN PENICILLIUM NOTATUM TO THE YIELD OF PENICILLIN IN SURFACE CULTURE

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One of the important factors bearing on the increased commercial production of penicillin has been the successful selection of strains of the *Penicillium notatum-chrysogenum* group that have superior penicillin-producing ability (Raper and Alexander, 1945; Gailey *et al.*, 1946). Of almost equal importance is the maintenance of these strains in a state of maximal penicillin production. A number of workers, Foster *et al.* (1943), Waksman and Reilly (1944), and Raper and Alexander (1945), have observed and reported the tendency of *P. notatum* to yield decreasing amounts of penicillin upon serial subculture. We have found one strain of this fungus to lose its penicillin productivity with such regularity that study of the phenomenon seemed warranted. With no name for this phenomenon in common use we have chosen to call it "penicillin run-down."

Methods for minimizing the "run-down" of penicillin-producing cultures have been published (Raper and Alexander, 1945; Foster *et al.*, 1943), but quantitative data have not been presented on the rate and extent of penicillin run-down and on conditions influencing its occurrence. It is the purpose of this paper to describe experiments pertaining to penicillin run-down in *P. notatum*, NRRL 1249.B21[1] and to describe our efforts to increase the penicillin yield of this strain by a program of selection and testing.

METHODS

Testing procedure. All isolates were tested for penicillin production in surface culture. Wide-mouthed, rectangular glass bottles[2] of about 2-liter capacity, 90 by 125 mm in cross section and 250 mm high, were filled with 300 ml of a medium of the following composition: $NaNO_3$, 3.0 g; $MgSO_4$, 0.014 g; KH_2PO_4, 0.50 g; $ZnSO_4 \cdot 7H_2O$, 0.0356 g; lactose monohydrate, 40.0 g; corn steep liquor, 80.0 ml; tap water to 1,000 ml; and an initial pH of 4.0 to 4.3.

This medium was recommended to us by Dr. A. J. Moyer of the Northern Regional Research Laboratory in Peoria and differs only slightly from the "Improved medium" of Moyer and Coghill (1946*a*). Following steam sterilization, cooling, and seeding by one of the techniques described below, the bottles were stacked on their sides in an incubator at 24 C for the entire fermentation cycle.

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[1] This culture was obtained from Dr. Kenneth B. Raper of the Northern Regional Research Laboratory, Peoria, Illinois.

[2] This bottle was fabricated by the Owens-Illinois Glass Company especially for use in the penicillin industry.

Growth first appeared in about 1.5 to 2 days, and pads of mycelium were usually completely formed on the third day. Daily samples were taken aseptically from beneath this mycelial pad, usually starting on the fifth or sixth day of the cycle and continuing until the peak had been detected, usually the ninth to eleventh day. Samples from six replicate bottles were pooled to give a measure of the daily performance of each isolate. The pH of each sample was measured with a glass electrode. Penicillin content was assayed by a hollow-cup agar plate method, adapted to the assay of penicillin by Dr. J. F. Norton of these laboratories. Each assay was based on the average of four inhibition zones, converted to Oxford units per ml by comparison with the daily standard curve.

Selection of isolates. Single germinated spores or separate colonies of *P. notatum* were obtained by streaking a dilute suspension of conidia on a petri plate of Czapek's solution agar (Thom and Raper, 1945). The streaked plates were incubated at a temperature of 24 C for 24 hours or for 7 days. In a few of our experiments, after 24 hours of incubation the streaked plate was placed under a dissecting microscope. Single germinated conidia were cut from the agar and transferred to plates of Czapek's solution agar for further growth. In the majority of our experiments, however, the conidia on the streaked plates were allowed to develop into week-old colonies. Each colony was assumed to have been derived from a single conidium.

In selecting single colonies for further study, a conscious effort was made to pick colonies which varied from one another in their macroscopic appearance and thus to include in our experiments as large a variety of colony types as could be obtained. A mass of conidia was transferred from each colony to duplicate slants of Czapek's solution agar. One of the agar slants was retained as a stock culture, and the other was used in obtaining a suspension of conidia for the inoculation of bran cultures.

Preparation of seed. Bran cultures were prepared by wetting 10 g of wheat bran in a 500-ml Erlenmeyer flask with an equal weight of sporulation solution containing 0.1 per cent asparagine and 3 per cent glycerol in tap water. Sterilization was effected by steam at 120 C for 30 minutes. The bran was inoculated with 1 ml of a conidial suspension and thoroughly shaken once a day until sporulation was completed. The conidia were washed off the bran with sterile water and used for the inoculation of our penicillin fermentation bottles.

Methods of seeding. "Hand-shaken seed" was prepared by selecting from a previous lot a fermentation bottle the pad of which showed no signs of contamination, adding 6 ml of sterile cottonseed oil as a flotation agent, and hand-shaking the entire bottle until the pad was well broken up and the conidia were loosened. Approximately 8 ml of this heavy spore suspension were used to seed each fermentation bottle of the subsequent lot. This corresponds to the type of mass spore transfers which is thought to produce heterokaryotic vigor in some fungi (Baker, 1944*a*, 1944*b*).

"Blended seed" was prepared by transferring aseptically all of the contents of a fermentation bottle or shaker flask to a sterile blender jar with special splash plate, described by Savage and Vander Brook (1946). Blending for 2 minutes at 10,000 rpm produces sufficient fragmentation for easy pipetting of the slurry. Blending for longer time intervals did not significantly increase the number of mycelial fragments. One ml of blended seed per bottle was a very adequate seeding rate when spores were present. When spores were almost or completely absent, the volume of blended seed had to be increased to 10 ml per bottle for adequate seeding. Nonsporulating mycelium for blending was produced in submerged culture by growing it serially in 500-ml Erlenmeyer flasks containing 100 ml each of a corn steep, lactose medium[3] of the following formula: lactose, 25.0 g; corn steep liquor, 80.0 ml; $CaCO_3$, 2.0 g; and $ZnSO_4 \cdot 7H_2O$, 0.044 g. Continuous agitation was provided by a shaking machine of the oscillating type with 95 complete 4-inch strokes per minute.

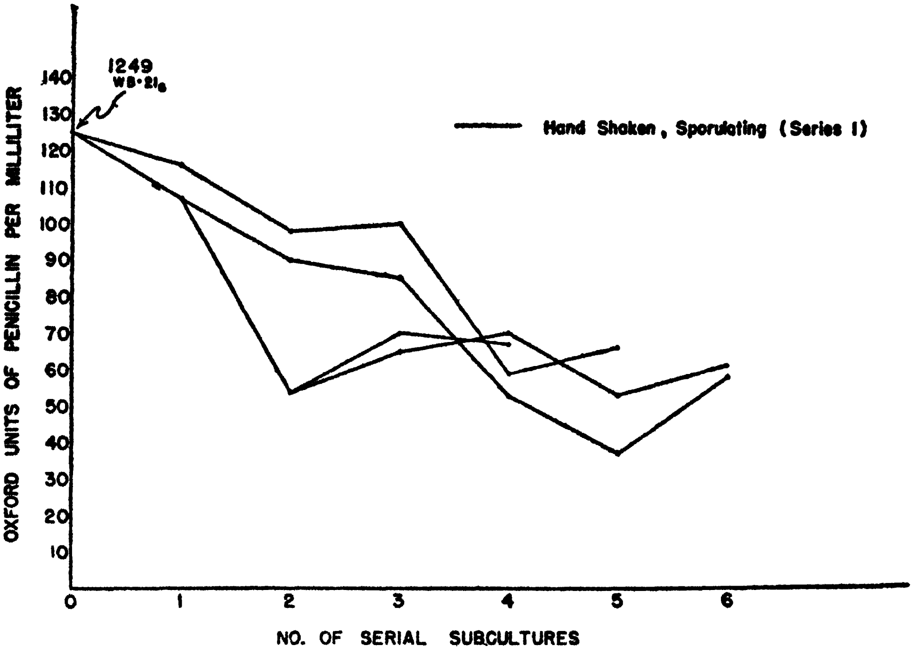

FIG. 1. THE RUN-DOWN UPON SERIAL SUBCULTURE OF A SPORULATING SEED OF PENICILLIUM NOTATUM NRRL 1249.B21

EXPERIMENTAL RESULTS

For some time we repeatedly observed that serial seeding with hand-shaken sporulating surface growth would result in a steady decline in the penicillin productivity of the strain from approximately 140 units to approximately 50 units per ml at the end of 5 to 7 serial generations. A typical set of performance curves for this mass transfer type of seeding with sporulating surface seed is shown in figure 1. Branching of the curves indicates an increase in the number of replicate lines being followed to give more data. Additional evidence of run-down may be seen in the quadruplicate or more controls of sporulating, hand-shaken, surface seed which were run as controls in figures 2, 3, and 4. On all of these first four figures the potency value plotted is the maximal beer potency reached by the particular line of seed of the designated generation. All factors known to influence potency, other than seed characteristics, were kept constant. The fermentation curves with hand-shaken seed were not followed after run-down had been established, since previous experience had shown repeatedly that no improvement would occur. Mycelial pads had become much heavier, tougher, and more rigid. Sporulation was much faster and heavier. The beer was clear and free of mycelial fragments. These characteristics of "run-down" seed showed no tendency to be reversible.

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[3] This formula is very similar to and no doubt derived from one of the corn steep, lactose formulas of Moyer and Coghill (1946b). We obtained it from the Lilly Research Laboratories, Indianapolis.

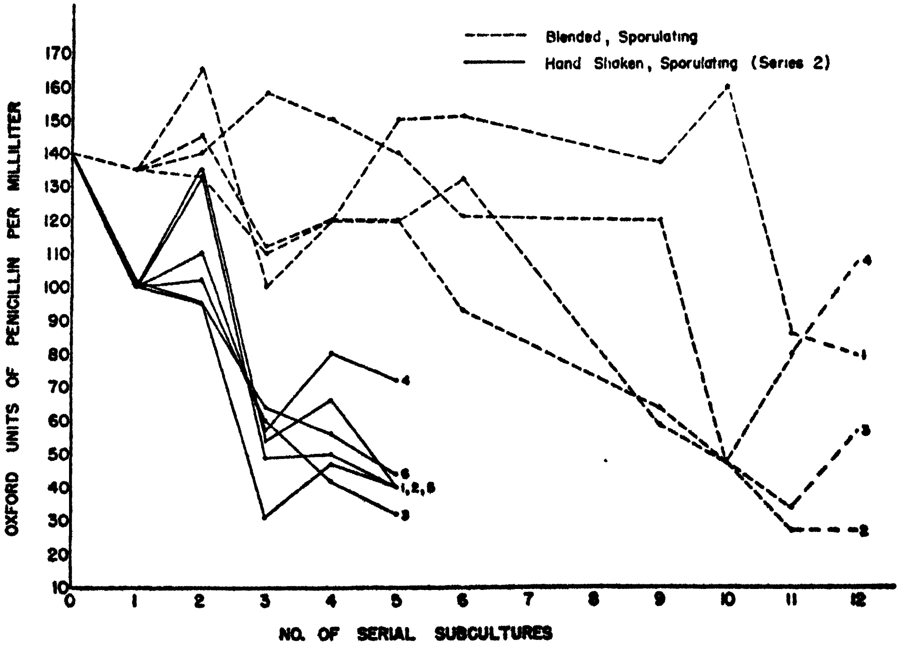

FIG. 2. A COMPARISON OF THE RUN-DOWN OF BLENDED AND HAND-SHAKEN SPORULATING SEEDS

In hand-shaken seed the predominant seed unit is the spore, with spores outnumbering mycelial fragments by at least one thousand to one. Blending the pad of mycelium and spores for 2 minutes increased the number of mycelial fragments enormously without changing the number of spores present. Therefore, a much greater percentage of the mycelium in the next generation was derived from a mycelial fragment than would have been the case with hand-shaken seed. A comparison of rates of run-down when these two types of seeding were used is shown in figure 2. Decreasing the percentage of spores in the seed caused the run-down to be delayed, although it did not prevent it. At the fifth generation, when 5 of 6 lines of hand-shaken, sporulating seed had already dropped to between 32 and 43 Oxford units per ml, all four of the blended seed lines were still producing 125 units per ml or more.

In the next experiment we tried to produce a seed completely free of spores by blending the pads as soon as a heavy mycelial pad was formed, but before the appearance of any colored spores could be detected. The usual set of four control lines was serially seeded with sporulating, hand-shaken seed to demonstrate run-down with the same starting seed. A comparison of the rates of

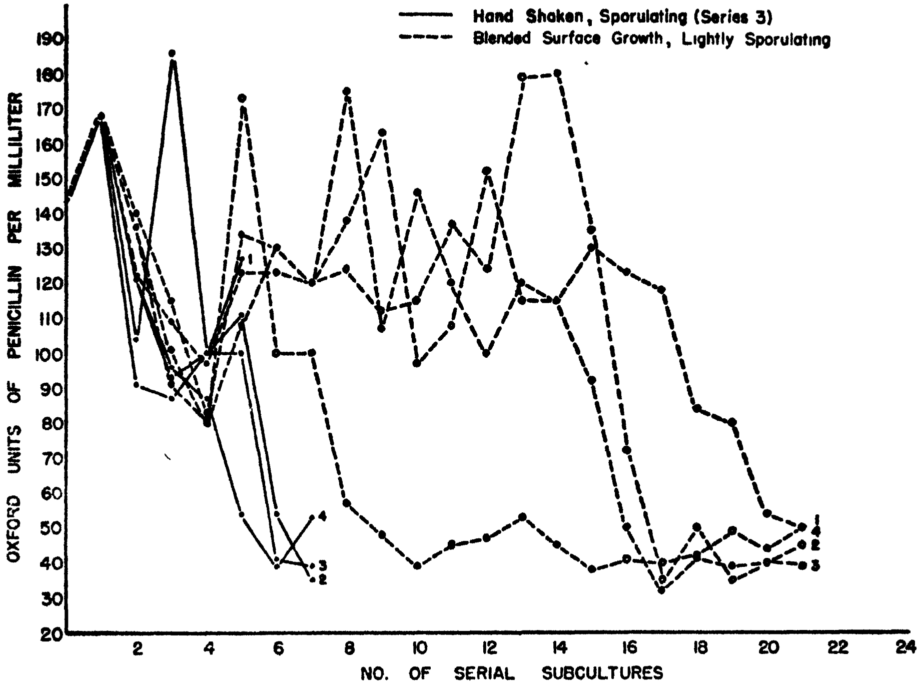

FIG. 3. A COMPARISON OF THE RUN-DOWN OF BLENDED, LIGHTLY SPORULATING SEED WITH HAND-SHAKEN SPORULATING SEED

run-down when these two types of seed were serially transferred is shown in figure 3.

Although we tried to produce a spore-free seed, in practice we found this was almost impossible to accomplish with this technique. On the second day, e.g., the mycelial pad might be too underdeveloped for blending. By the morning of the third day, the pad might be sufficiently heavy, but sporulation could already be seen. For this reason it is imperative that this seed be called "lightly sporulating" seed instead of nonsporulating seed. With it, run-down was still further delayed. As shown in figure 3, three out of four lines of the blended, lightly sporulating seed had run-down after 17 serial transfer generations, and after 20 generations all four lines had dropped to 50 units or less. All four lines of hand-shaken, sporulating seed were run-down after 6 generations.

Our second attempt to produce a nonsporulating seed for serial seeding consisted of growing the fungus in submerged culture serially, but doing all testing of this seed in surface culture. A surface growth would form in the shaker flasks along the wall above the liquid line, but this was shaken down into the liquid medium once each day to prevent any sporulation of surface mycelium. A comparison of four performance curves of this spore-free seed with the usual four control lines of sporulating, hand-shaken seed is shown in figure 4.

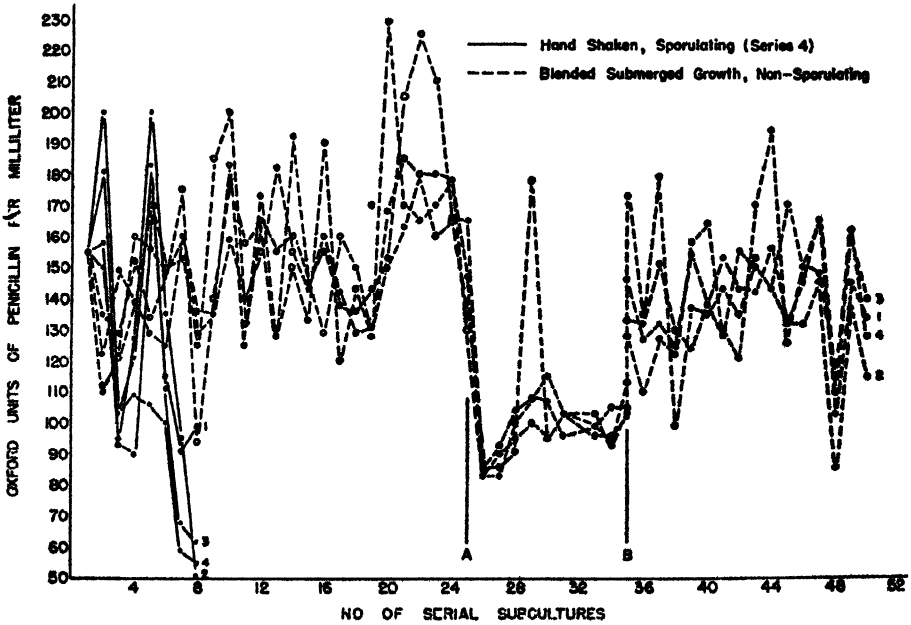

FIG. 4. A COMPARISON OF THE RUN-DOWN OF BLENDED, NONSPORULATING SEED WITH HAND-SHAKEN, SPORULATING SEED

At the 25th generation, a change in lot of corn steep caused all lines to drop together at the point indicated on figure 4 by the letter "A." From "A" to "B" (35th generation), this inferior lot caused all yields to be low. When the cause of this sudden drop was detected and a new lot of corn steep was used, all lines returned to their previous high potency levels. The experiment was finally terminated after all four lines remained at their original performance levels for 50 successive transfer generations. At this time the surface pads produced from this seed had all the gross characteristics of the original strain. Mycelium from the 50th transfer generation was blended and plated out from three dilutions of each of the four lines. Inspection of over 20 such plates containing a total of more than 200 colonies revealed no morphological changes from the parent seed.

Parallel with our studies of run-down, we have attempted to make use of the natural variation of *P. notatum* with the hope of obtaining higher-yielding strains through a course of continuous selection of the highest-yielding clone (h.y.c.) from among a number of isolations. Our experience with this technique is summarized in figure 5, in which all of the h.y.c.'s are arranged in a line. Each h.y.c. in turn is plated out for the selection of the next h.y.c. Continuous selection appears to maintain the strain at its original level. Although several isolates gave definitely lower penicillin levels and in gross appearance seemed to have mutated, no strain was encountered with a definitely superior ability to produce penicillin.

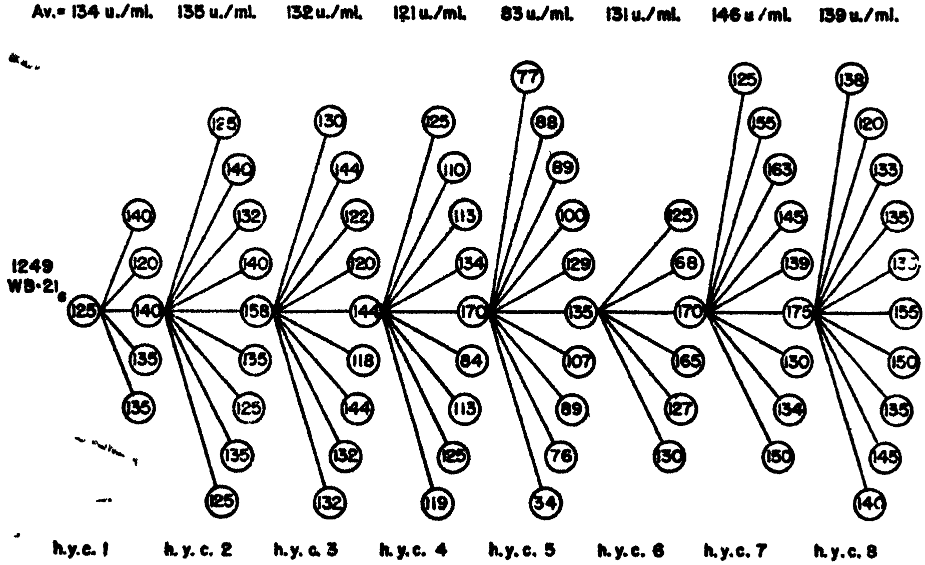

FIG. 5. THE CONTINUOUS SELECTION OF HIGHEST PENICILLIN-YIELDING CLONES OF PENICILLIUM NOTATUM

Numbers in circles refer to peak yield in Oxford units per ml. Each h.y.c. becomes the parent strain for selection of the subsequent h.y.c.

Our experience with the phenomenon of run-down led us to attempt a reversal of the phenomenon by means of the h.y.c. selection technique. At the end of a run-down series, we plated out the last sublot as a test of strain homogeneity. The result of several such sublot purifications is shown by a histogram in figure 6. Each sublot was found to contain a variety of strains which differed in color of conidial mass, texture of mycelial pad, and rate of sporulation. Some strains were recovered which appeared to be almost identical with the parent strain in both appearance and penicillin yield. Other strains differed in many respects from the parent strain. These new different strains were apparently both homogeneous and rather stable, for when we attempted to recover higher-yielding clones from them by the technique of h.y.c. selection, we failed to build up the penicillin yield of any daughter strain. This series is called the *Build-Up highest-yielding clone* series (BU h.y.c.) and the results are shown in figure 7.

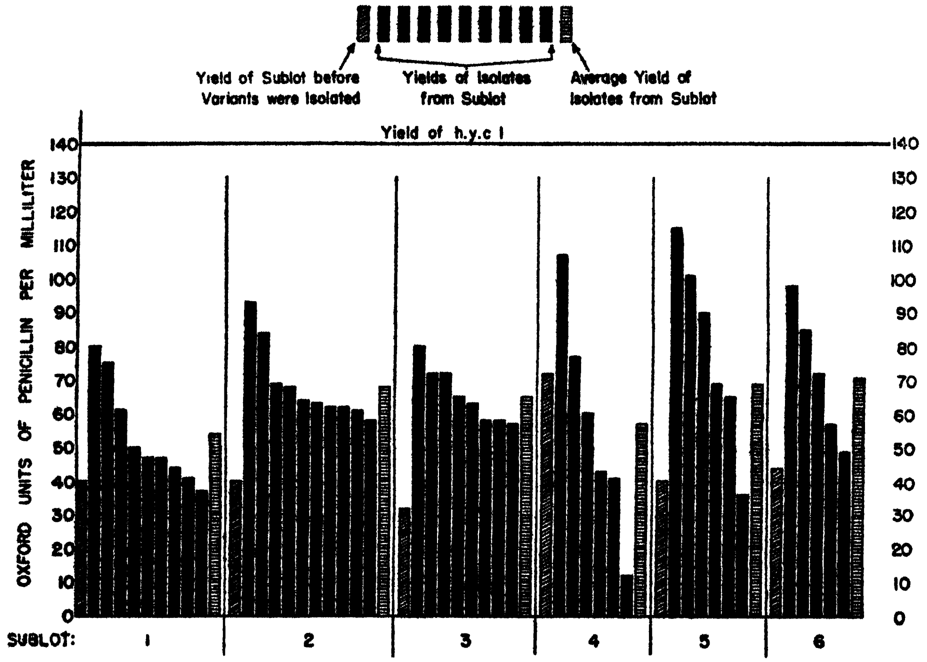

FIG. 6. PENICILLIN YIELD OF SIX RUN-DOWN SUBLOTS AND ISOLATES SELECTED FROM THEM

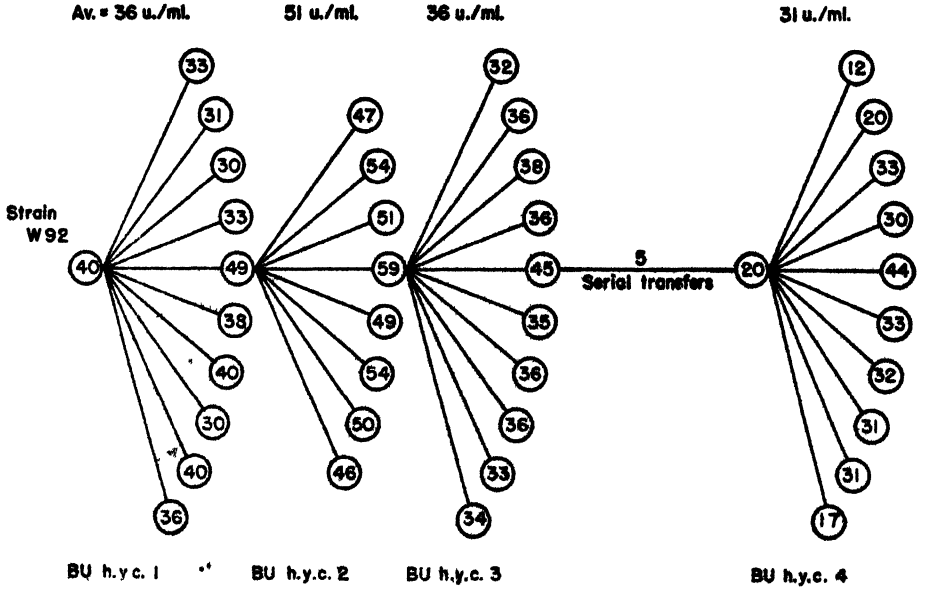

FIG. 7. THE CONTINUOUS SELECTION OF HIGHEST-YIELDING CLONES, STARTING WITH A LOW-YIELDING STRAIN

In order to increase the extent of variation by allowing a longer period of growth between isolations, we passed BU h.y.c. 3 serially through 5 successive transfer generations on the surface fermentation medium, along four separate lines. The end sublots were plated out and 100 clones were obtained by fishing single germinated spores and transferring each to the center of a plate of Czapek's solution agar. Seventy of these plates were uncontaminated. These were plug-assayed by the method of Raper *et al.* (1944). The ten strains giving the largest zones of inhibition were tested on fermentation medium and their peak yields recorded for the selection of BU h.y.c. 4, as shown in figure 7. None of these isolations was superior to the strain from which it originated.

DISCUSSION

Instead of finding that mass spore transfers maintain good production of penicillin by a high-yielding strain of *Penicillium notatum*, we have found that the fungus undergoes rapid natural variation with the production of mutants which usually sporulate both more rapidly and more heavily than the parent type. Run-down probably occurs whenever a mutation which decreases the yield of penicillin occurs simultaneously with a mutation which increases the degree of sporulation.

Suppression of sporulation suppresses run-down; and to the extent to which our experiments were carried, elimination of sporulation eliminated run-down. This suggests some sort of association between the process of sporulation and the occurrence of run-down. Whether mutants of the type observed to appear during run-down are actually produced more readily at the sporulation stage, or whether in the absence of sporulation these mutants are prevented from outgrowing the parent type is dfficult to assess at this time.

Quite regardless of any explanation for it, we have found that vegetative transfers are much better than spore transfers for preventing run-down of a strain. This is not in agreement with the conclusion of Foster *et al.* (1943) that "this degeneration can be eliminated by reducing vegetative transfers" and that serial transfers, when unavoidable, should be by means of spores.

Although the data which have been presented were collected on run-down in fermentation medium, all of our observations during the course of the work indicated that run-down occurs very regularly on other media as well, such as Czapek's medium and bran medium.

Low-yielding, heavily sporulating mutants, once they appear, rapidly outgrow the parent type. If serial subculture has not been continued too long, i.e., not more than 5 to 10 transfer "generations," without purification, it may be possible to isolate the parent type. The low-yielding mutants which appear are stable and show no tendency to reversion.

The nonsporulating type of mutants producing cottony white secondary colonies upon the pad were repeatedly observed. At no time, however, were they observed to overgrow the pad, even on subsequent serial transfer of the mixture of types.

SUMMARY

A high-yielding strain of *P. notatum* when serially subcultured was found to undergo rapid natural mutation, if sporulation was allowed to occur. Mutants which sporulated heavily and produced low yields of penicillin rapidly outgrew the parent type. This led to a decrease in penicillin productivity which we have called "penicillin run-down." When sporulation was prevented, this run-down did not occur at any time during 50 serial transfer generations.

Continuous selection of highest-yielding clones from a high-yielding strain did not result in the isolation of superior strains. Low-yielding mutants derived from this high-yielding strain were much more stable than the parent. Attempts to build up the penicillin yield of one of these low-yielding mutants by continuous selection were unsuccessful.

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STUDIES ON PENICILLINASE

III. THE EFFECT OF ANTIPENICILLINASE ON PENICILLIN-RESISTANT ORGANISMS

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Numerous reports (Carpenter *et al.*, 1944; Spink *et al.*, 1945; Tainter, 1945) have been recorded in the literature describing increased resistance of bacteria to various chemotherapeutic substances, including antibiotics. Increased resistance of organisms to penicillin has been observed (Spink *et al.*, 1945; Tainter, 1945; Demerec, 1945). In the latter case, attempts have been made to control infections caused by resistant organisms either by increasing the dosage of penicillin or by substituting another antibiotic or drug (Spink *et al.*, 1945; Wolnisky and Steenken, 1946).

Methods for preventing the development of resistance or for increasing the susceptibility of resistant organisms may be developed if the cause for this phenomenon can be determined. One of several possible factors involved in such resistance is the production, by certain organisms, of penicillinase, an enzyme capable of destroying penicillin. A correlation between the production of penicillinase and resistance to penicillin has been recorded in some cases (Kirby, 1944). In other resistant species, however, no penicillinase could be detected (Bondi and Dietz, 1944). Spink and Ferris (1945) found penicillinase in cultures made resistant to penicillin *in vivo* but none in those made resistant *in vitro*. These studies indicate that the production of penicillinase is not the sole factor in resistance, but it may well play an important role in certain instances. The possibility of enzyme inhibition by specific antibody (Sevag, 1945) was made use of by Perlstein and Liebmann (1945*a*), who produced antipenicillinase-immune serum and demonstrated that it protected penicillin from destruction by penicillinase (Perlstein and Liebmann, 1945*b*). No such results were obtained using normal serum. They postulated the formation of a penicillin, plasma protein complex which protects penicillin *in vitro* from destruction by penicillinase. Actually, such a theory is not necessary since the simple combination of antigen (penicillinase) and antibody (antipenicillinase) should prevent destruction of penicillin. The question also arises why antipenicillinase serum proteins should have an affinity for penicillin different from that of normal serum. All antibodies studied to date are globulins, and penicillin combines with normal albumin but not normal globulin (Chow and McKee, 1945). Even the albumin-penicillin complex proved to be active.

EXPERIMENTAL

Production and measurement of antipenicillinase. Rabbits were immunized to dialyzed penicillinase, assaying 1:1,024 (Housewright and Henry, 1947), by intravenous injection of 2 ml on alternate days for the first week and 3 ml on alternate days for the following 5 weeks.[3] Ten days after the last injection blood was withdrawn from each animal by cardiac puncture and the antibody response of the separated serum measured by precipitin tests and inhibition tests. In the precipitin test, sera were diluted twofold in series with saline, and an equal volume of penicillinase (1.0 ml, assay 1:1,024) was added. This mixture was incubated for 2 hours at 37 C and placed at 8 C overnight. Results were read the following morning.

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[1] With the technical assistance of A. S. Herring.

Precipitation occurred in serum dilutions through 1:56 but not in higher dilutions. No precipitation was observed in the antigen or serum controls. It cannot be stated from these data that the precipitin test was specific since the antigen used was admittedly impure.

TABLE 1

Inhibition test in which the antipenicillinase was diluted

ANTIPENICILLINASE DILUTION (A)	PENICILLINASE ASSAYING (B)	PENICILLINASE ASSAY AFTER COMBINING (A) AND (B) FOR 1 HOUR AT 37 C*
1:28	1:64	0
1:56	1:64	0
1:112	1:64	0
1:224	1:64	1:8
1:448	1:64	1:8
1:896	1:64	1:16
1:1,792	1:64	1:64
1:2,584	1:64	1:64
1:28	0	0
0	1:64	1:64

* These values have been corrected for the increase in volume resulting from a combination of equal volumes of (A) and (B).

Inhibition tests were performed either by holding the penicillinase concentration constant and diluting the sera, or by keeping the concentration of sera constant and diluting the penicillinase. Such mixtures were incubated for 1 hour at 37 C and then assayed for penicillinase (Housewright and Henry, 1947). Undiluted normal and immune rabbit sera inhibited growth of the test organism (*Bacillus anthracis*) used in the penicillinase assay. All inhibition tests, therefore, were performed using sera diluted at least 1:10, which was found to be beyond the growth-inhibitory range of either serum.

The results of a typical inhibition test in which the antigen was kept constant and the serum diluted are shown in table 1. The enzymatic action of penicillinase (assay 1:64) was lost completely after contact for 1 hour at 37 C with immune serum dilutions as high as 1:112. A partial loss was observed with serum dilutions of 1:224, 1:448, and 1:896.

Typical results of an inhibition test in which the antigen was diluted and the serum dilution was constant are shown in table 2. The activity of penicillinase assaying 1:64 was inhibited completely by a 1:28 dilution of antiserum, that of penicillinase assaying 1:128 was partially inhibited. No such results were obtained with normal rabbit serum in either inhibition test. There was no loss in enzyme activity when the enzyme, horse serum, rabbit antihorse serum, and saline were mixed and allowed to stand for 1 hour at 37 C.

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[3] Others were immunized by the subcutaneous injection of 1.0 ml of penicillinase at 4-day intervals for 8 weeks.

More direct evidence of the antigen antibody combination was obtained by use of the highly specific manometric assay for penicillinase (Henry and Housewright, 1947). This assay depends on the formation of penicilloic acid from penicillin, the reaction being catalyzed by penicillinase, resulting in increased acidity of the penicillin molecule. The reaction is allowed to proceed in bicarbonate buffer equilibrated against CO_2, and the change in acidity is reflected by the release of CO_2 which is measured manometrically. The effect of antipenicillinase on the above reaction is shown in figure 1. It is seen that penicillinase (assay 1:128) incubated at 37 C for 1 hour with antipenicillinase (1:10) lost about 90 per cent of its activity. Antipenicillinase began to combine with the enzyme almost immediately when it was introduced after allowing the penicillin-penicillinase reaction to proceed for 15 minutes. Controls indicated that there was no CO_2 retention by the concentration of serum used.

TABLE 2

Inhibition test in which the enzyme was diluted

ANTIPENICILLINASE DILUTION (A)	PENICILLINASE ASSAYING (B)	PENICILLINASE ASSAY AFTER COMBINING (A) AND (B) FOR 1 HOUR AT 37 C*
1:28	1:4,096	1:4,096
1:28	1:2,048	1:2,048
1:28	1:1,024	1:1,024
1:28	1:512	1:512
1:28	1:256	1:256
1:28	1:128	1:32
1:28	1:64	0
1:28	0	0
0	1:4,096	1:4,096

* These values have been corrected for the increase in volume resulting from a combination of equal volumes of (A) and (B).

Effect of antipenicillinase on the sensitivity of bacteria to penicillin. It has been demonstrated that, in certain instances (Spink *et al.*, 1945), naturally resistant organisms and those made resistant *in vivo* produce penicillinase, whereas sensitive organisms and those made resistant *in vitro* do not produce penicillinase. Table 3 shows the penicillin sensitivity of a number of organisms[3] in the presence of normal and antipenicillinase-immune sera.

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[3] *Staphylococcus aureus* Long IIIA, made resistant *in vivo*, and the sensitive Long I strain were obtained from Dr. W. W. Spink, of the University of Minnesota. The *Proteus* strains were obtained from Dr. M. G. Sevag of the University of Pennsylvania and the *Bacillus cereus* strain B-569 from Dr. R. D. Coghill of the Northern Regional Research Laboratories, Peoria, Ill.

Tube dilution assays (Housewright and Henry, 1947) revealed that only two of the organisms, *Staphylococcus aureus* Long IIIA and *Bacillus cereus* B-569, produced extracellular penicillinase assaying as high as 1:64 after 48 hours' incubation. It has been shown (table 1) that antipenicillinase diluted up to 1:112

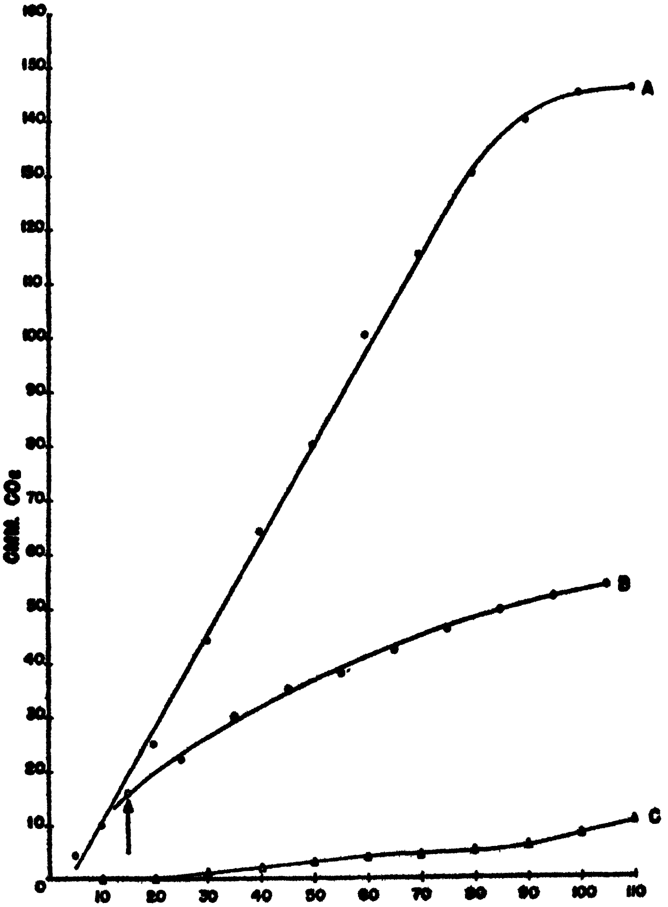

Fig. 1. Manometric Measurement of Penicillinase Activity in the Presence of Antipenicillinase

A. Penicillinase assaying 1:128 plus normal rabbit serum (1:10).

B. Penicillinase assaying 1:128; immune rabbit serum (1:10) tipped into main space of the vessel at 15 minutes.

C. Penicillinase assaying 1:128 and immune rabbit serum (1:10) incubated at 37 C for 1 hour prior to Warburg experiment.

inactivated penicillinase produced by *B. cereus* B-569 assaying 1:64. Antipenicillinase produced by the injection of penicillinase elaborated by *B. cereus* B-569 inactivated penicillinase produced by *S. aureus* Long IIIA. Thus penicillinase from these two sources are immunologically similar. If penicillin resistance is due wholly or in part to the production of extracellular penicillinase, then the presence of antipenicillinase in dilutions of 1:112 or less should increase the penicillin sensitivity of *S. aureus* Long IIIA and *B. cereus* B-569.

In order to investigate this point, the penicillin sensitivity of four sensitive and four resistant organisms in the presence of 1:14 normal and immune sera was determined. All tests were done in phenol red glucose broth (Difco) and in nutrient broth with commercial penicillin sodium (Pfizer, lot 585 C). Although it has been reported (Grollman, 1925; Kimmig and Weselmann, 1941; Robinson and Hogden, 1941) that phenol red combines with plasma proteins, no difference was observed in the results with the two media. As shown in table 3, the sensitivity to penicillin was increased by the presence of antipenicillinase only in those organisms producing extracellular penicillinase. These organisms, however, did not acquire a sensitivity sufficient to class them as penicillin-sensitive organisms.

TABLE 3

The effect of antipenicillinase on the sensitivity of bacteria to penicillin

ORGANISMS TESTED	MINIMAL INHIBITING CONCENTRATION OF PENICILLIN IN u/ml IN PRESENCE OF		EXTRACELLULAR PENICILLINASE	INTRACELLULAR PENICILLINASE
	Normal rabbit serum 1:14	Immune rabbit serum 1:14		
Penicillin-resistant				
S. aureus, Long IIIA	30	15	+	+
E. typhosa	50	50	−	+
Proteus vulgaris	25	25	−	+[*]
B. cereus, B-569	100	50	+	+
Penicillin-susceptible				
S. aureus, Long I	0.15	0.15	−	−[†]
S. aureus, 612	0.08	0.08	−	?
Proteus vulgaris	0.50	0.50	−	+[*]
B. anthracis, 99	0.04	0.04	−	?

[*] Reported by Bondi and Dietz.

[†] Reported by Spink and Ferris.

DISCUSSION

It appears that little would be gained by the clinical use of antipenicillinase in infections by penicillin-resistant organisms. Mixed infections may constitute an exception. A case of cutaneous anthrax that recently came to the attention of the authors demonstrated this possibility. The patient received a course of penicillin, which eliminated the bacteremia. *Bacillus anthracis* persisted, however, in the cutaneous lesion, contrary to the usual response to penicillin therapy (Ellingson *et al.*, 1946). Bacteriological examination revealed a mixed infection of *S. aureus* and *B. anthracis*. It was found that the anthrax organisms possessed the usual sensitivity to penicillin (0.02 to 0.04 u per ml), whereas the *S. aureus* was resistant (2.5 u per ml) and produced considerable quantities of extracellular penicillinase. Thus *B. anthracis* was protected from penicillin by the penicillinase produced by *S. aureus*. In such a case it is quite possible that *B. anthracis* could be eliminated by local packs of penicillin plus antipenicillinase. It may be worth while to evaluate the clinical use of antipenicillinase in such instances.

As mentioned in the introduction, one of the mechanisms of protection of an organism from penicillin is the production of penicillinase. It has been shown that this enzyme is produced intracellularly and is also found in certain instances extracellularly. The observation has been made in this work that antipenicillinase increases the penicillin sensitivity only of those organisms producing extracellular penicillinase. Since contact, or at least close proximity, must be established in order that an enzyme may act on a substrate, it is reasonable to postulate that the failure of antipenicillinase to increase the sensitivity of these organisms, in which only intracellular penicillinase is found, is due to the inability of antipenicillinase to pass the cell membrane. This inability of the antibody to pass the cell membrane could be explained by its molecular size alone. This hypothesis assumes that the site of action of penicillin is inside the cell, although actually no direct evidence as to the site of penicillin action is available. In view of these considerations, it is doubtful that an antiserum of higher titer would give different results. If the validity of this hypothesis is assumed, the question is still open as to how much penicillin resistance can be accounted for by intracellular penicillinase when present. Certainly other mechanisms must be considered, since neither extracellular nor intracellular penicillinase has been demonstrated in certain penicillin-resistant organisms (Bondi and Dietz, 1944).

SUMMARY

Antipenicillinase-immune serum was obtained by the injection of rabbits with penicillinase.

The combination of penicillinase and antipenicillinase and the consequent loss of activity of the former was demonstrated by inhibition tests and by the highly specific manometric method of assaying penicillinase.

The sensitivity of a number of organisms to penicillin was determined in the presence of normal serum and antipenicillinase-immune rabbit serum. Sensitivity to penicillin was increased only in those organisms which produced extracellular penicillinase.

It appears that antipenicillinase-immune serum would be of limited value as a chemotherapeutic adjunct, except possibly in the case of certain mixed infections.

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NOTES

SALMONELLA MONSCHAUI—A NEW TYPE

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Received for publication October 24, 1946

A new type of *Salmonella*, *S. monschaui*, was isolated from an infected wound of a patient in Walter Reed General Hospital. Although it is difficult to assess the role of this organism in the infected wound, its persistence over a long period of time would indicate that it played some part. The wound did not heal until after the organism had been eradicated.

The patient suffered a traumatic amputation of the foot when he stepped on a "schu-mine" in a heavily manured field in Monschau, Germany, on February 7, 1945. On his arrival at Walter Reed General Hospital, the stump was infected. Cultures taken on May 17, 1945, yielded a gram-positive bacillus, a diphtheroid organism, *Aerobacter aerogenes*, *Escherichia coli*, *Staphylococcus aureus*, and an unidentified gram-negative rod. Cultures were repeated and by November 10, 1945, the wound was healing but still carried *Staphylococcus aureus* and the unidentified gram-negative rod. The culture was sent to the Division of Bacteriology, Army Medical School, for identification.

The new type is highly pathogenic for mice, the MLD of *Salmonella monschaui* for 16- to 18-gram Swiss mice being 10 organisms when suspended in 5 per cent mucin. This is of the same order of virulence as freshly isolated strains of *Eberthella typhosa*.

Antigenic analyses were made following the methods of Edwards and Bruner (Kentucky Agr. Expt. Sta., Circ. 54). Alcohol-treated suspensions of the organism were agglutinated by *Salmonella adelaide* O serum (factor XXXV), but by no other sera. Absorption of *S. adelaide* O serum with *S. monschaui* left no residual agglutinins for *S. adelaide*.

Examination of the H antigens revealed that *S. monschaui* was monophasic. A motile culture was agglutinated to titer with *Salmonella oranienburg* H serum (factor m, t . . .). Single factor sera for factors m and t and reciprocal absorption tests demonstrated that the H antigen was characterized by the possession of the factor m, t . . . .

SUMMARY

Salmonella monschaui, a new type, has the antigenic formula XXXV m, t . . .— and an MLD for white Swiss mice of ten organisms when suspended in 5 per cent mucin.

THE PRODUCTION OF APPARENT CYCLES IN BACTERIAL VARIATION[1]

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Received for publication October 25, 1946

In the discussion of a recent report on dissociation in *Brucella abortus* (Braun: J. Bact., 52, 243) some results were mentioned which illustrate how competition between spontaneously arising mutants with different selective values can produce the appearance of successive, orderly changes. In view of the importance which has been attached to apparently cyclic changes in certain interpretations of bacterial dissociation, it appears desirable to place the actual data on record.

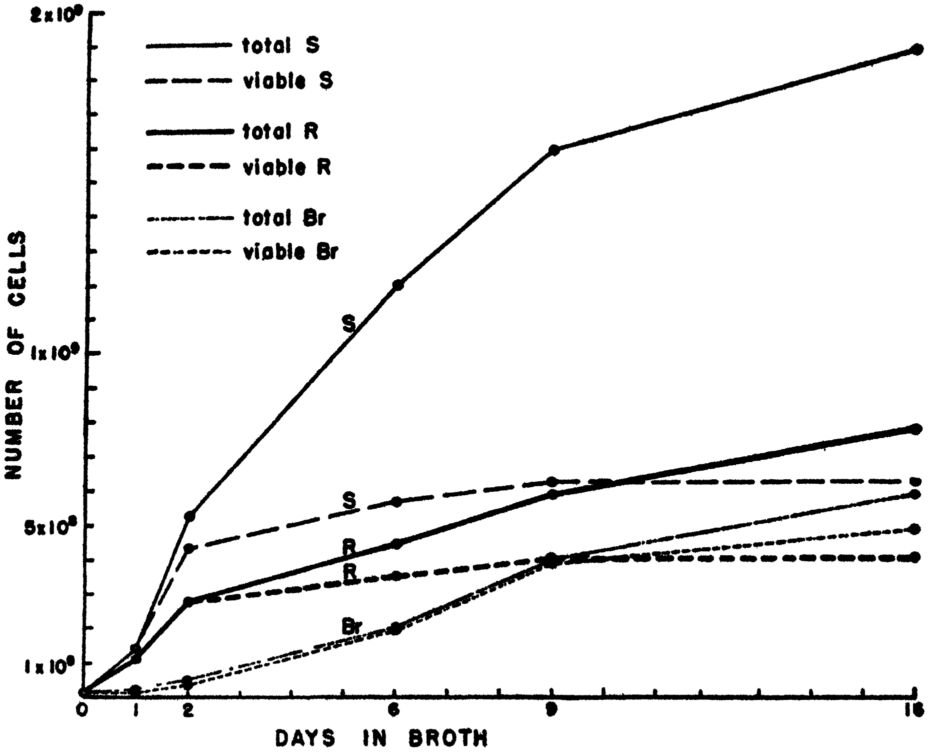

FIG. 1. NUMBER OF TOTAL AND VIABLE CELLS IN BROTH CULTURES OF S, R, AND BR TYPES OF BRUCELLA ABORTUS ON VARIOUS DAYS AFTER STARTING CULTURES

S, R, and Br (mucoid) types were isolated simultaneously from a 10-day-old broth culture that originally had been inoculated with members of one S clone.

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[1] This work has been supported by the Bureau of Animal Industry, U.S. Department of Agriculture, under co-operative agreement with the Regents of the University of California.

Each type was subsequently inoculated into individual broth cultures, and the numbers of viable and total cells were determined on various days after starting the cultures. The results, representing averages of 4 cultures for each type, are shown in the accompanying figure. It can be seen that S has the fastest growth rate but a low viability (i.e., ratio of total number of cells to viable number of cells per ml of broth); R has a far slower growth rate but rather high viability; and Br has the slowest growth rate but the highest viability. From these data it could be easily calculated that, when these types are competing within one population, the higher viability of the R types will suffice to permit their establishment at the cost of S types among the limited viable population. The Br types, with their even higher viability, will establish themselves eventually at the cost of both S and R types, but because of their slower growth rate it would take some time before Br types could become predominant. Thus, merely by the spontaneous appearance of mutant types differing in growth rates and viability, an apparently successive population change from S → R → Br can be produced if the cultures are maintained under conditions which allow continued reproduction.

THE INFLUENCE OF PRETREATING BACTERIA WITH ANIONIC AGENTS ON THE ANTIBACTERIAL ACTION OF CATIONIC GERMICIDES

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Received for publication November 13, 1946

Baker *et al.* (J. Exptl. Med., **74**, 621) have demonstrated that phospholipids prevent the inhibitory action of cationic germicides on bacterial metabolism when they are added to the bacterial suspension, either before or simultaneously with the surface-active cation. The effect persists even after the bacteria exposed to the phospholipids have been thoroughly washed. Valko and DuBois (J. Bact., **47**, 15) have shown that sodium dodecyl sulfate ("duponol PC") could, under certain conditions, reverse the antibacterial action of surface-active cations. Several other authors have likewise demonstrated the antagonistic influence of soap on these compounds.

We have investigated the action of alkyl dimethyl benzyl ammonium chlorides (ADBAC) on *Staphylococcus aureus* which had previously been treated with a soap or anionic detergent. The procedure was as follows: 0.5 ml of a 24-hour culture of *S. aureus* was treated with 5 ml of a 1:3,000 solution of sodium oleate for 10 minutes. The suspension was centrifuged, the supernatant fluid was decanted, and the bacteria were resuspended in 5.0 ml sterile water. Five-tenths ml of a 1:300 solution of ADBAC were then added (1:3,000 final dilution), and subcultures made after 5, 10, and 15 minutes. Growth was observed in the subcultures made after 5 and sometimes 10 minutes, but never after 15 minutes. A 1:3,000 concentration of ADBAC, under the same conditions of test, shows no growth in subcultures after only 1 minute. Therefore, it was apparent that the adsorbed soap had somewhat retarded the action of the cation.

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[1] Present address: Fuld Bros., Baltimore 31, Maryland.

Further results were obtained by a modified procedure which involved taking a 1-ml sample, diluting it with 9 or 99 ml water, transferring 1 ml of the solution to 10 ml of tryptone glucose extract agar, and incubating. In this case, both sodium oleate and sodium dodecyl sulfate were used. The results, again, demonstrated that sodium oleate retarded the action of ADBAC for up to more than 10 minutes. However, all the organisms were killed after 15 minutes. With sodium dodecyl sulfate no such effect took place, and there was no growth after 5 minutes.

From the above, it is clear that although prior treatment of the bacteria with anionic compounds delays the action of cationic germicides, it does not prevent it. These results are somewhat at variance with those of Baker *et al.* This difference is probably due to the fact that they used a concentration of about 1:20,000 ADBAC, whereas ours was about 1:3,000.

The following mechanism accounts for these observations. The surface-active cations first combine with the surface-active anions on or in the bacteria. This portion of the cations is inactivated. The cations must first reach the surface-active anions, which may have penetrated the bacteria to varying extents, and only then can the excess cations exert antibacterial action.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

INDIANA BRANCH

Terre Haute, Indiana, October 18, 1946

Interference Between the Cambridge Swine Strain of Influenza and the PR 8 Human Influenza Virus.

M. Michael Sigel, Virus Section, Second Army Area Medical Laboratory, Fort Benjamin Harrison, Indiana.

In 1943 we demonstrated interference between the Cambridge and PR 8 influenza virus strains. This interference phenomenon has been studied further, and certain quantitative relations have been established. In chicken embryos, the Cambridge virus prevented the growth of the PR 8 strain when the two were in approximately equal concentration in the inoculum. If the amount of the PR 8 virus exceeded that of the Cambridge in the inoculum (10 to 100 times), the former only multiplied. Under certain conditions, evidence of multiplication of both viruses in the same embryos was obtained.

Interference was also observed between the Cambridge strain and the Lee influenza virus.

A Method of Inoculating Embryonated Eggs for the Propagation of Rickettsia prowazeki.

H. A. Dettwiler and F. A. Darling, Lilly Research Laboratories, Indianapolis.

In the usual method of inoculation of incubated eggs the shell is punctured at the large or air-sac end, the inoculating needle thrust downward past the embryo, and the inoculum deposited in the region of the yolk sac. In the present method the hole is made in the small or tip end, the needle passed through the albumen, and the inoculum placed in the area surrounded by the yolk sac.

A comparative study of the two methods, involving approximately 35,000 eggs in each group, indicated that tip-end inoculation resulted in a slight reduction in the number of unusable yolk sacs from dead embryos and markedly increased the quality of the yolk sacs available for harvest. As determined by microscopic examination of stained films, 32 per cent of the group inoculated by the usual method graded "good to excellent" in rickettsial growth, whereas in the group inoculated through the tip end, 68 per cent were of that degree of quality.

Estimation of Biological End Points by Semilogarithmic Graph Paper.

Frank Streightoff, Lilly Research Laboratories, Indianapolis.

The Reed-Muench method is widely used in computing biological end points, such as the LD_{50} of immunized and control mice in testing pertussis vaccine. In the Reed-Muench method data are tabulated and the percentage death rate determined at each dosage level. With a two cycle semilogarithmic graph paper, dosage levels are represented logarithmically on the ordinate and the percentage death rate on the abscissa. The first points in the tabulation, above and below 50 per cent death rate, are located on the graph and connected by a straight line. The LD_{50} dosage is determined by inspection.

Semilogarithmic graph paper eliminates several calculations, and leaves a record which can be checked at a glance.

On the Relation Between Penicillin Resistance and the Production of Penicillin Inactivators in Staphylococcus.

S. E. Luria and Rachel M. Arbogast, Indiana University, Bloomington, Indiana, and Carnegie Institution, Cold Spring Harbor, New York.

The individual cells of penicillin-inactivating (penicillinase-positive) strains of *Staphylococcus* often prove sensitive to penicillin when tested by plating small inocula in penicillin agar (Luria). Simple penicillin resistance, not accompanied by penicillinase production, has been shown to originate by spontaneous mutations in sensitive strains (Demerec). Similar mutations to resistance occur in the cells of penicillinase-producing strains. The origin of penicillinase-positive strains from negative strains *in vivo* in the course of penicillin therapy, assumed by many workers, appears doubtful in view of the following findings: (1) No penicillinase production appears when sensitive strains are made resistant *in vitro*. This is also true for strains supposed to have given penicillinase-positive variants *in vivo*. (2) No increase in penicillinase production occurs when penicillinase-positive strains are grown in penicillin media. (3) Penicillinase-positive strains are very frequent in first isolates from untreated patients. (4) Mixtures of positive and negative strains are found in the same untreated lesion.

It is suggested that the apparent origin of positive strains in the course of therapy may be simulated by selection of positive strains out of unrecognized mixtures with negative ones in the same patient. It is, however, possible that mutations from penicillinase-negative to positive strains occur, which show up *in vivo* but not *in vitro* because of differences in selection pressures in different environments.

IMPROVING STREPTOMYCIN YIELDS BY STRAIN SELECTION AND INOCULUM DEVELOPMENT.

Alfred R. Stanley, Research Department, Commercial Solvents Corporation, Terre Haute, Indiana.

Yields of streptomycin can be increased manyfold by strain selection. An original culture giving assays of 100 to 200 μg per ml was developed by strain selection to give 400 to 500 regularly. After irradiation of this culture with ultraviolet light, colony selection gave cultures in which yields of 600 to 800 μg per ml were not uncommon, with one strain giving an assay of over 900 in shake flasks. This selection was accomplished by isolating large numbers of single colony cultures of *Streptomyces griseus* and testing them for streptomycin production. Those giving the best yields were subcultured for plant use. These comparative tests must be run on the fermentation medium being used in the plant, since the highest-producing cultures on one medium are not always the highest on another.

Antibiotic yields can also be increased by selecting the inoculum medium that gives the highest assays in the fermenter. If the wrong medium is used for culturing the inoculum, low yields will be obtained in the fermenter.

NUTRITION OF STREPTOMYCES GRISEUS IN RELATION TO STREPTOMYCIN TITER.

Ralph E. Bennett, Commercial Solvents Corporation, Terre Haute, Indiana.

In conjunction with pilot plant production of streptomycin, the nutrition of *Streptomyces griseus* was studied in attempts to secure the optimum medium. Media reported in the literature, such as corn steep water and peptone, beef extract and peptone, and soy bean, were found with a selected strain of *S. griseus* to give routinely streptomycin titers of 400 μg per ml in laboratory shake flasks and 200 to 400 μg per ml in 300-gallon pilot plant runs. Other commercially available materials which gave comparable titers in the laboratory were acid-hydrolyzed casein, acid-hydrolyzed rabbit fur, acid-hydrolyzed wheat gluten, acid-hydrolyzed stillage from wheat mash yeast alcohol fermentation, and asparagus butt juice concentrate.

Media containing an abundance of proteins, preferably in the form of amino acids or polypeptides, are a prerequisite for high titers. A comparison of several media before and after acid hydrolysis or enzyme digestion shows that the digested forms of nutrient materials are much the more favorable.

Corn steep, beef extract, or some substitute must be used with most of the protein-bearing materials mentioned above. The use of corn steep or beef extract without special precautions yields a final streptomycin containing dangerous amounts of histamine. Much of the stimulation due to steep water can be obtained by inclusion in the medium of a proper concentration of steep ash or other potassium-bearing salts, such as potassium phosphate or chloride.

ISOLATION OF A PURE CULTURE OF A CELLULOSE-DIGESTING BACTERIUM.

L. Y. Quinn, Bacteriology Department, Purdue University.

The culture studied by Murray (J. Bact., 47, 117) digested cellulose even in the presence of a stream of air. Murray recognized a variety of morphological forms in his culture, but he concluded they were all aerobes or facultative aerobes.

Pratt suggested that the cellulose decomposer might be an anaerobe growing with an aerobe which reduces the oxygen tension.

The present studies of this same culture have led to the isolation of the anaerobic organism or organisms which digest cellulose only at greatly reduced oxygen tension. After 21 days' incubation at 65 C, the morphological type consists of a gram-negative filament beaded with gram-positive granules at regular intervals. This organism was isolated on a completely synthetic medium containing Hungate's cellulose, and the digestion of cellulose was evident after 3 days' incubation. Digestion of cellulose began in a yellow band near the surface of the cellulose mass in broth cultures, but later the entire cellulose mass became diffusely yellow. Digestion stopped when the pH reached 5.55. Substances reducing Benedict's solution were produced.

USE OF KITTENS FOR DEMONSTRATION OF ENTEROTOXIN PRODUCTION BY STRAINS OF CLOSTRIDIUM PERFRINGENS IMPLICATED IN HUMAN FOOD POISONING. *Rafael Marinelareña and L. S. McClung,* Bacteriological Laboratories, Indiana University, Bloomington.

Preliminary studies indicate that the young kitten may be sensitive to enterotoxin production by *Clostridum perfringens* as evidenced by the production of diarrhea following feeding of culture supernatant after centrifugation of a young culture. Although in the assay of enterotoxin of staphylococcal origin vomiting is a more positive symptom, the absence of vomiting in our tests may not be significant since humans (volunteer and outbreak cases) usually did not display this symptom. Further studies are in progress.

FLAGELLAR AND SOMATIC AGGLUTINATION OF CLOSTRIDIUM BIFERMENTANS AND CLOSTRIDIUM SORDELLI. *Helen Michael and L. S. McClung,* Bacteriological Laboratories, Indiana University, Bloomington.

An intensive study has been made of the flagellar and somatic agglutination of a large collection of strains of *Clostridium bifermentans,* including historical cultures and others isolated during the recent war, and authentic strains of *Clostridium sordelli* from Hall, Sordelli, and Meleney. The results indicate antigenic identity of these cultures, since all strains would react in sera prepared from either organism and strains chosen at random could absorb all agglutinins from such sera. The data lend further support to the identity of the two organisms.

LECITHINASE PRODUCTION BY CLOSTRIDIUM HEMOLYTICUM. *L. S. McClung and Ruth Toabe,* Bacteriological Laboratories, Indiana University, Bloomington.

In addition to the species previously reported, *Clostridium hemolyticum* has been found to be an active producer of lecithinase as shown by the lecithovitellin reaction. In egg yolk plate cultures the zone of precipitation is larger than that of *Clostridium perfringens,* and a high titer is obtained on assay of supernatants following centrifugation of young cultures. Further studies are in progress on the relation of this factor to the hemolytic toxin for which the species is best known.

EASTERN PENNSYLVANIA CHAPTER

ONE HUNDRED AND EIGHTY-NINTH MEETING, PHILADELPHIA, PENNSYLVANIA, OCTOBER 22, 1946

INTRAVENOUS INFECTION OF THE CHICK EMBRYO WITH HUMAN TUBERCLE BACILLI: INHIBITORY EFFECTS OF STREPTOMYCIN.

Henry F. Lee and Abram Stavitsky, Children's Hospital, Philadelphia, Pennsylvania.

A new chick embryo technique was devised which made possible the rapid production of disseminated tuberculous lesions within the parenchymatous organs of chick embryos. The bacteriostatic effects of compounds may be tested in a short period of time in the presence of intact tissues. Studies utilizing streptomycin and certain other chemotherapeutic substances served to demonstrate the validity of the method. Streptomycin prevented the development of histologic evidence of infection in chick embryos intravenously inoculated with tubercle bacilli, but bacilli were recovered upon culturing the tissues.

REDUCING THE PYROGENICITY OF CONCENTRATED SOLUTIONS OF PROTEINS.

Robert B. Pennell and William E. Smith, Department of Immuno-chemistry, Medical Research Division, Sharp and Dohme Laboratories, Glenolden, Pennsylvania.

The use of Seitz filtration for the removal of pyrogenic substances from solutions of crystalloids and other solutes of comparatively small molecular weight has been common practice. The effectiveness of this procedure for the removal of pyrogens from protein solutions has been slight, however. The treatment of pyrogenic protein solutions with "decalso," an exchange silicate, particularly when used in conjunction with a new type of Seitz pad, Republic filters' K6 and S6 pads, is effective in the reduction, or complete removal, of pyrogenicity from such solutions. The "decalso" itself must receive a pretreatment, e.g., a carbonate wash, for removal of inherent pyrogens before it is used for treating protein solutions. For protein solutions of moderate concentration, 5 to 10 per cent, and low pyrogenicity, 30 grams of "decalso" and 60 sq inches of pad surface per liter of solution treated have been found effective. The amount of "decalso" and the pad surface per unit volume of solution treated increases with the pyrogenicity and the protein concentration of the solution. Except in extreme instances the foregoing considerations result in a procedure which is practical in the routine production of pyrogen-free protein solutions. The time of treatment with "decalso" was not shown to be of extreme importance. Attempts to demonstrate the manner of the pyrogen removal were unsuccessful.

SOME OBSERVATIONS ON MICROBIOLOGY IN THE U.S.S.R.

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METHYLCELLULOSE AND BACTERIAL MOTILITY

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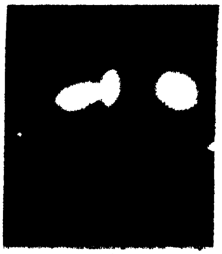
In a previous publication (1946) bacterial motility was shown to be due, not to activity of so-called "flagella," but to a gyrating and undulating movement of the bacterial body itself. "Flagella" are not motor organs but rather fortuitous appendages. The gyrating and undulating movement of the bacterial body originates in the protoplasm which lines the inner surface of the cell wall. It forces the cell wall into the shape of a moving spiral. As a result, the outer covering of the cell wall, which consists chiefly of polysaccharide material, is during fast movement, with the added effect of friction, mechanically twisted into a tail as in figure 1. This has been described, photographed, and filmed by me on previous occasions (1930, 1931–32, 1938, 1940, 1941*a*, 1941*b*, 1942). At other times this twisted material untwists into a varying number of wavy threads, which have so far gone under the name of "flagella." These twistings and untwistings have up till now only become visible with sunlight dark-ground microscopy (1938, 1940, 1941*a*).

All this not only fits in with modern conceptions of bacterial structure, as analyzed by Knaysi (1938, 1944) and Dubos (1945), but it supports them. A tough cell wall enclosing the protoplasm naturally bars this live substance from transmitting the required energy to supposititious "flagella" outside the cell wall. Even the electron microscope has not brought evidence that "flagella" pierce the cell wall, witness the comprehensive review of Mudd and Anderson (1944).

All protoplasmic energy must arise within the cell wall. Mechanically this energy reveals itself by throwing the cell wall into spirillar contortions, which result in movement in undulating and gyrating fashion. The outer covering of bacteria, referred to as the "slime layer" by Knaysi (1938, 1944), is largely polysaccharide material, and as such very plastic. It has long-chain molecules, with a tendency to form long micelles, and can be drawn out into threads several centimeters long (Knaysi, 1944). During rapid rectilinear movement it tapers automatically into a tail, as in figure 1. The tail may be very much longer than is shown in figure 1. Slowing down reveals its spiral nature, as in figure 2, as it is fashioned by the gyrating, undulating movement of the body. Occasionally it splits, as in figures 3 and 4, after which it may reunite again. Its general twisted structure becomes apparent on other occasions when I have seen it come apart in a number of fine wavy threads (1938). These are the supposed "flagella" for which so many staining processes have been invented, and which are now well known from electron microscope pictures, in which they lie as a tangled mass around the bacteria.

"Flagellum" both in bacteriology and zoology means a motor organ. As, however, both the tail and the wavy threads resulting from its untwisting owe



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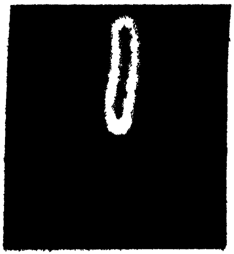

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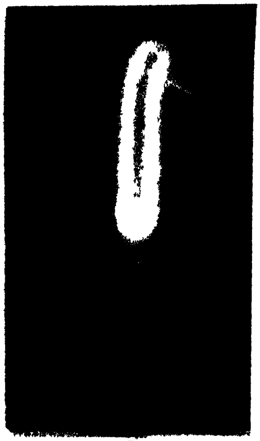

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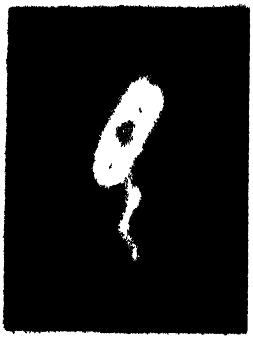

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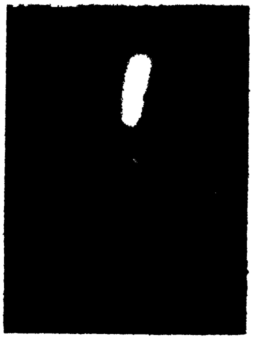

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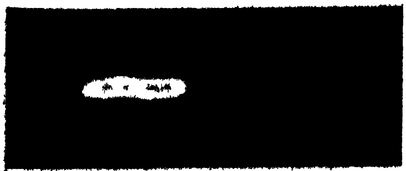

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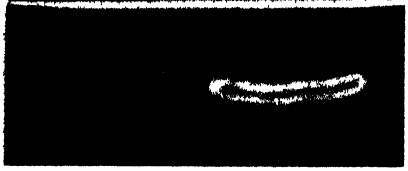

THE PHOTOMICROGRAPHS

Some of these have had to be made with sunlight, the others were made with a 100 CP pointolite lamp, but in no case do they illustrate more than what becomes visible when a 100 CP pointolite lamp is used, as stated in the text. It must be realized that overexposure of bacteria lying still or overprinting of negatives, which is sometimes necessary, leads to the appearance in the photomicrographs of thicker appendages than in reality exist.

Fig. 1. Bacterium with short straight tail. ×2,000.

Fig. 2 Bacterium slowing down, straight tail has become broadly wound coil. Note spiral shape of bacterium. ×4,000.

Fig. 3. Bacterium slowing down and splitting its broadly wound tail. Note spiral shape. ×5,000

Fig. 4. Bacterium with split tail. Note spiral shape. ×5,000.

their origin to the motility of the body, their function must be regarded as entirely passive, and the term "flagellum" should no longer be applied to any of these structures. At best the tail acts as a passive rudder, contributing to the steadiness of the gyrating undulating movement of the body. But neither tail nor "flagella" possess energy; they do not represent a driving force. These things should be designated as "polysaccharide twirls," a term which adequately sums up and describes their nature and origin.

It also follows that motile bacteria should no longer be regarded and described as "rods." They may look rodlike in killed, fixed, and stained preparations, although even then curved shapes occur and have often been noticed. For systematic classification purposes, however, this rodlike shape should no longer be accepted as correct. Bacterial morphology should be based on the shape which becomes manifest during active life, which here means motility. "Motile bacteria" thus becomes a self-contradictory expression. When "bacteria" are motile, they move in undulating gyrating fashion and exhibit spiral shape, and not the appearance of rods, or "bacteria." This upsets bacterial classification, but facts must be recognized, and their recognition will eventually lead to clarification and simplification of taxonomy. In this paper the word "bacteria" is used without prejudice.

Different species of bacteria are credited with differences in the number and the attachment of their "flagella." Serology relies to a certain extent on differences in the physicochemical nature of the polysaccharide covering of bacteria. Such physicochemical differences may well express themselves in the number and situation of the polysaccharide twirls that become unwound, and have given rise to the terms, "peritrichic," "lophotrichic," etc.

POLYSACCHARIDE TWIRLS

Abolishing the word "flagella" in bacteriology and replacing it by a term like "polysaccharide twirls" does not rob the structures referred to of all importance. They remain curious morphological phenomena, and may even retain some taxonomic interest. Motility, however, is not dependent on their presence, nor development. Very motile bacteria need not exhibit any, as I have shown (1946). Their development depends on the production of a good "slime layer" of polysaccharide material. The haphazard results of staining methods for "flagella" here find their explanation. Very fast moving bacteria do not necessarily possess much polysaccharide coating.[1]

The action of H-agglutinating sera needs further revision. I have shown

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[1] An illuminating story concerning Zettnow and "flagellar" staining in general is told by Levenson (1938). He was in a postgraduate class in which Zettnow taught his own method, but no member of the class succeeded in staining even a single "flagellum"!

FIG. 5. Bacterium with broadly wound coil attached to one side. ×4,500.

FIG. 6. Bacterium with tail in the shape of a broadly wound coil moving from the pole to the side. ×3,000.

FIG. 7. Bacterium with tail in shape of broadly wound coil definitely over to one side. ×3,000.

FIG. 8. Bacterium with tail in shape of broadly wound coil definitely attached to one side. ×3,000.

(1938, 1940) that such sera have no real agglutinating action. All they do is to cover "flagella" and body surface with a thick precipitate, presumably globulins, which stiffens the "flagella." This was confirmed with the electron microscope by Mudd and Anderson (1941). The stiffened "flagella" or "twirls" resemble corkscrews. This is the whole effect of the H-agglutinating serum. What then follows is *fortuitous* entanglement of the stiff twirls, caused by accidental currents and slight remnants of bacterial motility. "Flagellar agglutination," supposed to be the result of an H-agglutinating serum, does not exist; it is neither flagellar nor does the serum cause agglutination.

METHYLCELLULOSE

Methylcellulose is a water-soluble cellulose ether, produced and sold by the Dow Chemical Company of Midland, Michigan, as "methocel." According to the firm's booklet it forms colloid solutions, which are colorless, odorless, tasteless, and nontoxic. It is available in six viscosity types, ranging from 15 centipoises to 4,000 centipoises in a 2 per cent solution. Professor Robert Breed kindly supplied me with a quantity of methocel, with the suggestion that I should apply it to the study of bacterial motility, for which I here express my thanks. It was also through his kind help that, when I found it useful, the firm supplied me with larger quantities of the 15 cps and the 4,000 cps variety, for which I remain very grateful. Methocel has been of great assistance to me in elucidating the motility of bacteria.

EFFECT OF METHOCEL ON MOTILE BACTERIA

Most of my observations have been made on *Eberthella typhosa*, but control observations on *Proteus vulgaris*, *Bacillus megatherium*, *Bacillus cereus*, *Pseudomonas fluorescens*, and *Bacillus subtilis* confirmed that my conceptions of motility and "flagella" are applicable to most, if not all, motile bacteria.

The effect of methocel solutions on motile bacteria is twofold. Their viscosity slows down motility, and a slight precipitate which descends on the bacterial bodies and wavy appendages makes these last structures more easily visible.

Methocel solutions differ in their effect from the colloid solutions previously used by other authors for the purpose of slowing down motility through increasing viscosity (Neumann, 1925, 1928; Neumuller, 1927; Loveland, 1933; Wei, 1936). These authors used gelatin or gum, and the result was such a voluminous precipitate of these substances on the supposed "flagella" that they appeared as heavy corkscrews, the same as if they had been treated with an H-agglutinating serum, with similar end results (Pijper, 1930, 1931–32, 1938, 1941*a*). This precipitate was not noticed as such by the authors, and they regarded the thick wavy threads they saw attached to the bacterial bodies as plaits of otherwise normal "flagella." This misinterpretation added to the confusion. Such complete artifacts which have proved so misleading to previous authors do not usually occur in the weaker solutions of methocel, as employed by me, or if they do, they only occur after prolonged exposure.

Methocel solutions of suitable strength have the advantage of precipitating just so much material onto bacterial appendages that their slight thickening makes them visible by ordinary dark-ground microscopy with a 100 CP pointolite lamp, without having to take recourse to sunlight. The "polysaccharide twirls," formerly called "flagella," become visible in full activity, in a condition which closely approximates normality. The slight coating of methocel scarcely affects their behavior and appearance. Figures 1 to 8 show early stages. Figures 9 to 15 show various more or less advanced stages of "untwisting." For demonstration purposes the methocel solution technique is bound to supersede the elaborate and often disappointing fixing and staining methods, which at best show a mass of entangled wavy threads, without any clue as to function.

The slowing down effect through viscosity is the feature which elucidates bacterial movement. In my previous studies of bacterial motility I used to aim at the brightest possible light, which was provided by the sun, and the fastest possible bacteria, in order to get a good view of their tails. As a result their bodies became just a blur, and the nature of their movement became hidden. Placing such high-speed bacteria in a suitable methocel solution and using less brilliant light in the form of a 100 CP pointolite lamp completely altered the picture. The bacteria now moved slowly with or without tails or other appendages. Their bodies exhibited graceful, regular undulating and gyrating movements, which propelled them through the fluid (figure 4). The slower they moved, the more pronounced the undulations became (figure 16). Readily visible in long forms, the same kind of movement also became quite evident in the shorter and even very short forms (figures 21 and 22). Very fast swimmers moved for a time as very elongated spirals (figure 17), but as a rule adopted a more leisurely, broadly undulating gyrating movement later on (figure 18). The whole spectacle can scarcely be watched without the thought dawning upon the spectator that in this undulating gyrating movement is the force which propels the bacterium, and that it is not in the tail, which follows somewhat limply behind. Every bacteriologist has occasionally watched bacteria move across the field with a curiously oscillating movement. This "waddle" has so far not been recognized for what it is, viz., a gyrating undulating movement propelling the body in spiral form. The spiral shape of the moving bacterium in methylcellulose solution is quite unmistakable, and it persists when the bacterium comes to rest, or dies (figure 19). It is more persistent after death in methocel solutions than in watery solutions. Dividing forms also show it (figures 20 to 22).

In not too viscous solutions, in which speed is not too much reduced, many bacteria still drag a straight tail along. Slowing down and more pronounced gyration and undulation cause a broadening of the coils of the tail (figure 2). With a return to higher speed the tail may stretch again. If, during periods of rest or slower speed, there is only one appendage, it is usually attached at one end; but it may then go somewhat to one side (figures 5 to 8). When the tail untwists in methocel solutions, it usually at first splits into two wavy appendages, rarely three (figure 14). In methocel solutions the untwisting sometimes proceeds further than this, and on occasion the twisted polysaccharide material

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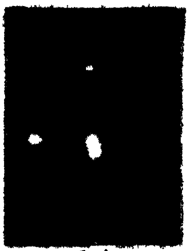

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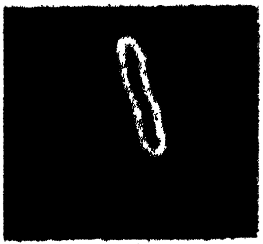

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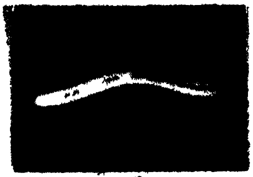

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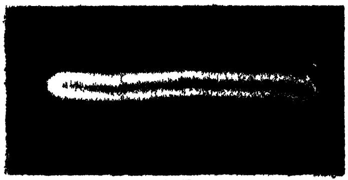

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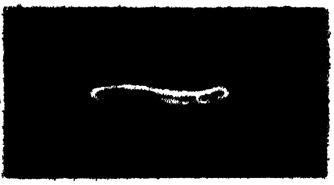

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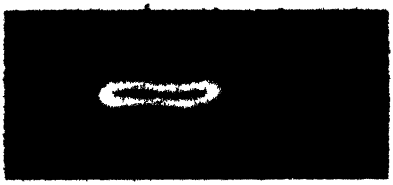

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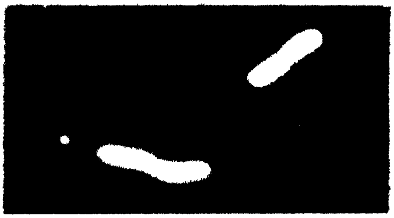

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FIG. 9. Bacterium with tail definitely split into two wavy appendages. × 3,000.

FIG. 10. Bacterium with two symmetrically arranged wavy appendages. × 3,000.

FIG 11 Bacterium with two irregularly arranged wavy appendages × 4,000

FIGS 12 and 13 Two bacteria, each with two irregularly arranged wavy appendages. × 1,500

FIG. 14 Bacterium with three wavy appendages. × 1,500.

FIG. 15. One bacterium of which the body is badly blurred in order to show at least 5 wavy appendages. × 4,000

FIG. 16. Two bacteria which have just divided, both showing spiral shape and together forming a larger spiral. × 2,000.

FIG. 17. Long bacterium showing drawn-out spiral shape. × 3,500.

FIG. 18. Bacterium showing perfect spiral shape. × 2,000.

FIG. 19. Dead bacterium still showing spiral shape. × 3,000.

FIGS. 20 and 21. See facing page for legends.

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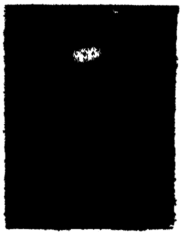

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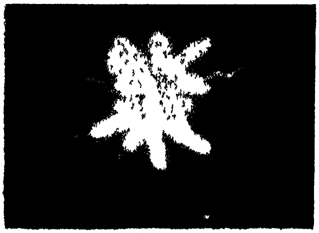

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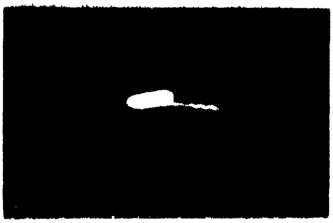

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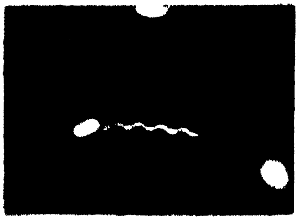

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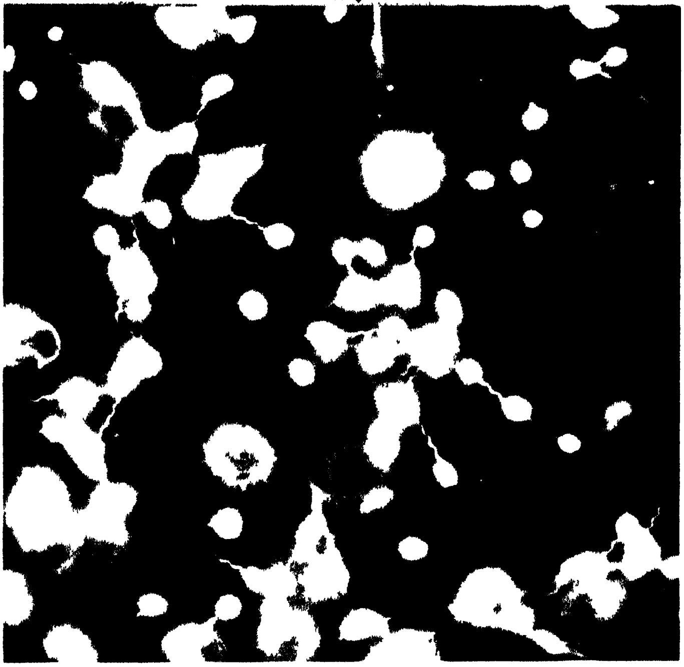

Fig. 20. Two bacteria which have just divided, the frontal one showing perfect spiral shape. × 3,000.

Fig. 21. Two sets of bacteria which have just divided. Note spiral shape of both sets. × 3,000.

Fig. 22. Short bacterium which has just divided and both the new individuals and the combination show spiral shape. × 3,000.

Fig. 23. Bacterium with one wavy appendage which ends in indistinct mass around bacterium. × 2,000.

Fig. 24. Clump of bacteria with wavy appendages sticking out. × 2,000.

Figs. 25 and 26. Two bacteria which have been in methylcellulose solution too long, with marked thickening of wavy appendages. × 2,000.

Fig. 27. Large number of bacteria, most of them slowing down or stopping suddenly and showing broadly coiled tails. × 2,000.

comes apart into several fine wavy threads, as in figure 15. I have, however, never seen in methocel solutions the very large number of wavy threads that I have seen in watery solutions, in which it may reach the number of 20 or thereabouts. The explanation probably is that the thin coating of methocel prevents complete deployment.

When the tail splits into just two appendages, which is shown in various stages in figures 4, 9, and 10, they may be arranged symmetrically, but often there is no regularity about their attachment (figures 11, 12, and 13).

In many instances the appendages stop short a little distance from the bacterial body, indicating that they really belong to the outer covering (figure 23).

In methocel solutions bacteria show a tendency to stick to one another once contact has been made accidentally. This leads to formation of small groups, the bodies becoming attached, while wavy threads stick out (figure 24).

TECHNIQUE AND DETAILED RESULTS OF OBSERVING MOTILE BACTERIA IN METHOCEL SOLUTIONS

Methocel solutions are best made by weighing out the required quantity, pouring on half the required saline at boiling temperature, allowing the material to soak for some minutes, and then adding the rest of the saline. The mixture, in a corked flask, is then placed in a refrigerator and left there until solution is complete. For the more concentrated solutions it is advantageous to invert the flasks from time to time. The solutions will be found to be perfectly clear to the naked eye, but for dark-ground work, where every blemish shows up, it is advisable to subject the thicker solutions to rapid centrifugalization, and the thinner ones to filtration through a Seitz filter. In a refrigerator solutions made under sterile conditions keep very well. I have occasionally noticed fungal growth; it is better to discard such flasks. In methocel solutions viscosity increases with a rise in temperature, and so at room temperature the viscosity is higher than in the refrigerator. It is probably further increased by the heat rays in the focus of the dark-ground condenser, but to what extent it is impossible to say. The amount of precipitation on bodies and appendages is probably also affected by the temperature, apart from the concentration of the solution. Then there is the time factor. As time proceeds the amount of precipitate always increases, and a bacterium that looks like figure 1 or 2 in a suitable solution of methocel may keep this appearance for several hours, but eventually it will look like figure 25 or 26, although 24 hours or more may be required to reach this stage. Taking all these uncontrollable factors into account, it is hardly feasible to prescribe the exact strength and kind of solution required for a particular effect.

Microscope preparations are made by placing a large loopful of the methocel solution on a slide and rubbing a very small loopful of broth culture gently into it. A cover slip is dropped on and sufficient time given for the drop to spread out to the edges. The cover slip is sealed down on two sides. An enclosed small air bubble is helpful for supplying oxygen; in its neighborhood bacteria live longer and remain more active.

A 100 CP pointolite lamp is sufficient to see everything that is illustrated in my photomicrographs. A small 5 amp. arc lamp does the same. I use a Zeiss cardioid condenser, but other good glass dark-ground condensers answer well. As an objective I like the 60× Zeiss, with iris, and a fairly strong ocular, say 15× or even 20× is indicated. Binoculars can be used, and when they are arranged to give a stereoscopic effect, the undulating gyrating movement becomes particularly obvious. All binocular attachments, however, decrease the amount of light reaching the eye, and finer details may become invisible this way unless the lamp is very bright.

It is essential to work with bacteria possessing a high degree of motility, and this can usually be achieved by making them grow in several passages through soft agar, either in a petri dish or through a U tube.

For the benefit of those wanting to repeat the experiments I shall now briefly give the results obtained in various methocel solutions, with the reservation that, for the reasons mentioned above, it is difficult to duplicate results. The notes refer to typhoid bacilli, strain Ty 901.

METHOCEL VISCOSITY TYPE FIFTEEN CPS

With *0.25 per cent solution*, absolute viscosity about 2.5 cps. Motility very good, with numbers of very long and thin tails, much longer than appears in figure 1. After half an hour some tails become broadly wound coils, as in figure 2 or 27, but motility remains good. An occasional one untwists and becomes like figure 9. Movement remains too fast to show undulation and gyration. After two hours motility decreases, and, although some very long and straight tails are left, many bacteria now show two appendages. These sometimes come together again and form one tail, which then again splits, thus illustrating the reversibility of the change.

With *0.5 per cent solution*, absolute viscosity about 3.5 cps. Motility very good, with numbers of long tails. Occasionally slight gyrating and undulating movement is visible, but on the whole movement is too fast. Many suddenly slow down with tail becoming a broad coil, as in figure 2 or 27, and then again return to high speed and a long thin tail. Collisions rather more frequent than is normal. Untwisting and retwisting of tails quite common. After a few hours: motility decreased, some swimming with broadly coiled tail; undulating gyrating movement visible here and there, but not pronounced.

With *1 per cent solution*, absolute viscosity about 6 cps. Motility at first quite good, with many long thin tails, and very little undulation and gyration. After some minutes broadly wound tails appear as in figure 2 or 27, which, however, often return to the elongated shape. Many bacteria in their straightforward course suddenly reverse frontal and caudal pole, while the tail remains in place. This means that they can turn a half somersault within their coat of polysaccharide combined with methocel material. Bumping against one another is not uncommon. After one hour: still many normal tails, although generally motility is much less and many broadly wound tails and double appendages have appeared. Many bacteria start swimming in narrow circles, swinging their tails around. After a few hours: little motility left, many broadly wound tails, moving and still. Some splitting of tails still occurs. No obvious undulation and gyration.

With *1.25 per cent solution*, absolute viscosity about 7.5 cps. Motility at first very good, but slows down fairly soon, and then undulating gyrating movement begins to show up (figure 4). Tails become broader spirals rather soon. Bumping occurs, and the individuals often stick. Appendages thicken visibly after a few hours and motility comes to an end.

With *1.5 per cent solution*, absolute viscosity about 9.5 cps. After initial high velocity with long tails, many sudden stoppages and reverses take place. Indications of undulating and gyrating movement start early (figures 16, 17, and 18). Circular movement with broadly wound tails fairly common. Tails split and reunite often. Clumps form easily, with wavy appendages sticking out, sometimes revolving erratically, probably as a result of tension in the material (figure 24).

With *2 per cent solution*, absolute viscosity about 15 cps. Very much the same as in the 1.5 per cent solution. Sudden "reverses" quite common.

With *2.5 per cent solution*, absolute viscosity about 24 cps. At first quite normal long tails, but the bigger bacteria begin to show undulating gyrating movement quite clearly, while the smaller ones often swim in narrow circles. Tails rapidly become broader coils and splitting is common. Thickening of appendages not more marked than in previous less viscous solutions.

With *3 per cent solution*, absolute viscosity about 37 cps. On the whole the same as in the 2.5 per cent solution, but there is more undulating gyrating movement. Motility stops rather soon. Thickening of appendages not more than in previous solutions.

With *3.5 per cent solution*, absolute viscosity about 54 cps. At first good motility with typical long tails, but undulating gyrating movement starts quickly and becomes quite manifest, while the tails rapidly become broad coils.

With *4 per cent solution*, absolute viscosity about 80 cps. After usual initial high velocity with long thin tails, undulation and gyration become quite marked and motility endures, some bacteria undulating and gyrating with long thin tails and others with broadly wound tails.

With *4.5 per cent solution*, absolute viscosity about 113 cps. Motility very poor except near air bubbles. Those that move show fair undulation and gyration with long tails that rapidly become broad coils.

With *5 per cent solution*, absolute viscosity about 160 cps. Scarcely any motility.

METHOCEL VISCOSITY TYPE FOUR THOUSAND CPS

With *0.1 per cent solution*, absolute viscosity about 7 cps. Very good motility with faintly visible but very long and straight tails.

With *0.25 per cent solution*, absolute viscosity about 20 cps. Very good motility, tails becoming more easily visible. Occasionally a tail becomes a broad coil. Sudden reversals in direction also become visible, with the tail sometimes left behind, and the bacterium then swims over its own tail, making it obvious that the body directs the tail and not the tail the body. Motility decreases with time, and broadly wound tails appear. There is then little gyrating undulating movement.

With *0.5 per cent solution*, absolute viscosity about 50 cps. Very good motility at first, with thin long tails, which rapidly become more easily visible. The longer forms begin to show gyration and undulation, but the curves of the bodies remain fairly flat, the speed being still fairly high.

With *0.75 per cent solution*, absolute viscosity about 140 cps. Motility is now much less from the start, and there is a good deal of undulation and gyration. Tails easily visible, sometimes still as long, straight structures, sometimes as broad coils. Tails are often left behind at sudden reversals in direction, and then appear again at the other end. Tails often extremely long. This medium is very good for showing definite gyration and undulation with tails attached. Bacteria lying still usually show definite spiral shapes.

With *1 per cent solution*, absolute viscosity about 300 cps. Motility is slow, there is very marked undulation and gyration, tails usually are broad coils. A few rapidly moving bacteria still show elongated, very straight tails. Undulation and gyration become quite universal after an hour.

With *1.5 per cent solution*, absolute viscosity about 1,200 cps. Undulation and gyration very common from the start, with slow motility, while tails gradually change from the elongated form to broad coils. Many bacteria reverse suddenly their direction and then swim over their tails, which appear again at the other end. After half an hour many clumps are formed, with coiled appendages sticking out.

With *2 per cent solution*, absolute viscosity 4,000 cps. Motility very slow, with very marked undulation and gyration, sometimes with a long, thin tail, which is often left behind during sudden reversals of direction. Motility stops fairly soon, but even after an hour there are still individual bacteria showing very clear undulation and gyration, with elongated tails.

With *2.5 per cent solution*, absolute viscosity 11,000 cps. Motility very limited and slow, but this is accompanied by very good undulation and gyration.

SUMMARY

Solutions of methylcellulose (sold as "methocel" by the Dow Company) provided a particularly suitable medium for the study of bacterial motility. Such solutions possess sufficient viscosity to slow down the movement of otherwise fast-moving bacteria and thus supply conditions for detailed observation of movement, which appears as if it were in "slow motion." The special advantage of methocel over the solutions of gelatin and gum used hitherto for this purpose is that the precipitation of the colloid material on bacterial bodies and appendages is minimal, so that motility is not hindered by this thickening. With gelatin and gum the amount precipitated is always so large that the bacteria, with enormously thickened appendages, appear as caricatures of themselves, and no valid conclusions can be drawn from such artifacts. With

methocel this precipitation and thickening is a very slow process and solutions can be prepared in which for a long time the amount precipitated on the bacteria is just enough to make the fine appendages ("flagella") visible by ordinary dark-ground methods with ordinary lamps, without having to take recourse to the brilliancy of sunlight and dark-ground techniques. Methocel therefore places the dark-ground study of bacterial motility and bacterial appendages during life within the scope of ordinary microscopic technique.

A study of motile bacteria under these conditions leaves no doubt that such bacteria propel themselves by means of undulating gyrating movement of their bodies, in which "flagella" play no active part. It becomes obvious that the wavy appendages which appear under these conditions and which used to be called "flagella" are just the product of motility and not its cause. They consist of the outer covering of bacteria, which is mainly polysaccharide, and through the undulating gyrating movement of the body they are twisted off in the shape of long spiral tails or more or less numerous thin, wavy threads.

This new conception of bacterial motility and of the nature of the so-called "flagella" has already been put forward, with all the evidence on which it is based, in a previous paper (Pijper, 1946). The present paper describes in detail the technique and the results achieved with various methocel solutions. On the whole, methocel of the 15 cps variety is more suitable for making the conduct of appendages visible, and methocel of the 4,000 cps variety brings out the undulation and gyration more readily, but both phenomena can be observed in suitable solutions of either variety.

Motile bacteria representing several groups have been examined, and they all exhibit these undulating gyrating or "spirillar" movements. It therefore appears that motile bacteria can no longer be regarded as rods, or "bacteria," but must be classed as something like spirilla. The term "flagella," which would indicate motor organs, will have to be dropped and replaced by an expression such as "polysaccharide twirls" or "mucous twirls."

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THE EFFECTS OF BACTERIA ON THE GROWTH OF TRICHOMONAS FOETUS (PROTOZOA)

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Very little work has been reported concerning the effects of bacteria on the growth *in vitro* of *Trichomonas foetus.* Numerous reports in the literature indicate that many trichomonad infections in cattle are accompanied by a varied bacterial flora. The influence these organisms may have on the ultimate establishment of bovine trichomoniasis is not known. The purpose of this paper is to present research concerning the effect of certain bacteria upon the growth of *T. foetus in vitro.*

Reidmüller (1928) isolated *Brucella abortus,* gram-negative rods, diplococci, and streptococci from aborted fetuses in which trichomonads were found. Abelein (1929), Weidenauer (1930), and Kohl (1933) found diplococci, streptococci, staphylococci, and diphtheroids in trichomonad pyometra fluid. Hahn (1935) and Wohlfarth (1937) identified *Corynebacterium pyogenes* in cases of trichomonad pyometra. Smythe (1943) and Murray (1943) also reported the coexistence of *C. pyogenes* and *T. foetus* in cattle.

Kerr (1942) pointed out that lesions in cattle infected with *T. foetus* may have been due to associated or concurrent secondary bacterial infections by such organisms as diphtheroids or streptococci. Kerr (1943) also recognized the presence of bacterial infection in an abscessed condition of the epididymis of a bull that had been inoculated with a diphtheroid-contaminated culture of *T. foetus.* Karlson and Boyd (1941) found *T. foetus* in the seminal vesicle of a bull with bilateral orchitis and vesiculitis; a pure culture of *C. pyogenes* was present in both seminal vesicles and the left epididymis. Patrizi (1940) reported on a case of genital tuberculosis accompanied by trichomoniasis in a bull.

Abelein (1929), Reidmüller (1928), and Gehring and Murray (1933) were unsuccessful in cultivating *T. foetus* free from bacteria. Witte (1933) was the first to cultivate a pure strain of this flagellate. He inoculated bacteria-free trichomonad pyometra exudate into serum broth cultures. Glaser and Coria (1935) described the "V" tube migration technique and isolated in pure culture the first American strain of *T. foetus.* Since their report, many methods of isolation have been suggested.

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It has been known for some time that *T. foetus* will not grow *in vitro* in the presence of certain contaminating bacteria. Morgan (1942) first demonstrated that an atypical strain of *Corynebacterium renalis* grows in close association with *T. foetus* since the flagellates multiplied more rapidly in its presence than in bacteria-free cultures. He believed that this acceleration may be caused by a change in the medium due to hydrolysis of proteins and carbohydrates by the diphtheroid, but the nature of this action is not known.

Plastridge (1943) was unable to grow *T. foetus* in the presence of staphylococci and coliform organisms. Zeetti (1940) found trichomonads could not grow with various species of micrococci. Williams and Plastridge (1946) found that *T. foetus* was completely destroyed in less than 24 hours by *Streptococcus viridans, Streptococcus faecalis, Streptococcus lactis, Escherichia coli, Aerobacter aerogenes*, and *Proteus vulgaris*, and in 48 hours by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Moderate growth occurred in the presence of *Micrococcus tetragenus, Sarcina lutea*, and *Bacillus subtilis*. These investigators and Morgan (1946) were successful in freeing trichomonad cultures of most contaminating bacteria by the use of penicillin and streptomycin.

MATERIALS AND METHODS

Culture medium. The culture medium employed was a modification of Schneider's (1942) citrate medium. This medium has proved to be very satisfactory for the growth of *T. foetus* and all of the bacteria studied in this work.

Schneider's modified medium was prepared in 2 portions: slant and supernatant. A citrate solution was first made and is of the following composition: sodium chloride, 5 g; magnesium sulfate, 0.2 g; ammonium phosphate (monobasic), 1 g; potassium phosphate (dibasic), 1 g; sodium citrate, 2 g; glucose, 10 g; and distilled water to 1,000 ml.

The slant portion of the medium was then prepared. Six fresh eggs were thoroughly mixed with 60 ml of citrated bovine blood and 75 ml of the citrate solution by agitation in a flask containing glass beads. This suspension was then filtered through cheese cloth, and 2.5-ml portions were tubed in 16-mm test tubes. The tubes were slanted and inspissated in an autoclave for 5 to 10 minutes. Air was not evacuated from the chamber during inspissation. When carefully done, smooth glistening slants resulted.

To prepare the liquid supernatant, 500 ml of the citrate solution were added to 50 ml of bovine serum, 8 ml of hematin solution (50 mg in 20 ml of distilled water), and 0.5 ml of a 1.6 per cent aqueous solution of bromcresol purple. This solution was then filtered through a double thickness of coarse filter paper and 5 ml were added to each slanted tube. The tubes were plugged with cotton, autoclaved for 30 minutes at 15 pounds' pressure, and stored in the refrigerator until used. The final pH of the medium was approximately 7.0 to 7.2

Organisms employed. The trichomonad employed in these studies was strain "BR", which was routinely maintained in the laboratory. It was originally isolated by Morgan and Wisnicky (1942) from a cow suffering from a trichomonad pyometra and has since been kept in pure culture on the modified Schneider's medium.

Cultures of *Corynebacterium hofmannii*, *Corynebacterium xerosis*, *Corynebacterium diphtheriae*, *Streptococcus hemolyticus*, *Streptococcus salivarius*, *Vibrio comma*, *Neisseria catarrhalis*, *Staphylococcus aureus*, *Staphylococcus albus*, *Shigella dysenteriae*, and *Shigella paradysenteriae* were kindly supplied by the Department of Medical Bacteriology, Medical School, University of Wisconsin.

Corynebacterium renale (atypical; this diphtheroid would not ferment glucose), *C. pyogenes*, *Streptococcus bovis* (5 strains), *Streptococcus faecalis* (2 strains), *Aerobacter aerogenes*, and *Escherichia coli*, all recently isolated from bovine pyometras, were obtained from the Department of Veterinary Science, College of Agriculture, University of Wisconsin. *Brucella abortus* (strain 19) was also supplied by this department.

Through the kindness of the Department of Agricultural Bacteriology, College of Agriculture, University of Wisconsin, cultures of *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Corynebacterium equi* were obtained.

An unidentified *Sarcina* was isolated by the writers from a sheath swab of an apparently normal bull used in an artificial insemination ring. This organism was nonmotile, gram-negative, did not liquefy gelatin or Loeffler's blood serum, fermented glucose, and formed a deep yellow pigment. Corynebacteria cultures 714, 154SH, 49, and RIDG5, and strains 161SH and 152SH of *Corynebacterium renale* were isolated by Morgan *et al.* (1946) from sheath swabs of apparently normal breeding bulls. Cultures B-1, B-2, and B-3 of diphtheroids were recovered by Morgan *et al.* (1946) from the seminal vesicle of a bull infected with *T. foetus.*

Determination of growth rates. The counting of *T. foetus* in the various cultures inoculated was accomplished with a Levy-Hausser hemocytometer counting chamber. The culture tubes were carefully rotated and samples withdrawn aseptically with a 6-mm wire loop.

All cultures in this work were incubated at 37 C. An inoculum of approximately 50,000 active flagellates per tube was standard throughout this study. Contamination was guarded against constantly and tested for periodically by the examination of films stained with Loeffler's methylene blue. Contaminated cultures were discarded and replaced by others in order to keep the series constant for direct comparisons. Tubes of freshly prepared uninoculated media were incubated for 24 hours at 37 C and examined for bacterial growth as an added precaution against contamination.

Before study of the effect of various bacteria, bacterial filtrates, and heat-killed bacterial cultures on the growth of this trichomonad, the normal growth rate of *T. foetus* employing a constant inoculum in a constant quantity of medium was determined. A total of 178 replicate cultures were counted to establish the normal growth curve of *T. foetus.*

In the experiments utilizing live bacteria, 5 tubes of Schneider's medium were inoculated simultaneously with trichomonads and a loop of a 24-hour bacterial culture was grown in the same medium. *Brucella abortus* (strain 19) and *C. renale* (strain 152SH) were incubated for 48 hours before inoculation since 24 hours was not sufficient to permit adequate growth.

Studies with killed bacteria were made on 24-hour cultures which were grown in Schneider's medium and heat-killed in a 60 C water bath for 1 hour. After cooling, 5 tubes of the heat-killed cultures were inoculated with *T. foetus* for each species of bacteria tested. The effect of killed bacteria on the growth of this trichomonad was determined with the following bacteria: *C. renale* (atypical), *C. pyogenes*, *C. hofmannii*, *C. xerosis*, *C. diphtheriae*, *Streptococcus hemolyticus*, *S. salivarius*, *S. bovis* (5 strains), *S. faecalis* (2 strains), *V. comma*, *Neisseria catarrhalis*, *Staphylococcus aureus*, *S. albus*, *Shigella dysenteriae*, *S. paradysenteriae*, *A. aerogenes*, and *E. coli*.

Seitz filtrates of 24-hour bacterial cultures were prepared from the liquid portion of Schneider's medium. These filtrates were aseptically pipetted into tubes containing only the inspissated blood-egg slant and inoculated with *T. foetus*. Five such tubes were inoculated for each species of bacterium studied. Filtrates were made from the following bacteria: *C. renale* (atypical), *C. hofmannii*, *C. xerosis*, *C. diphtheriae*, *S. bovis* (strains 2 and 4), *S. faecalis* (strain 1), *S. hemolyticus*, *S. salivarius*, *Shigella dysenteriae*, *Shigella paradysenteriae*, *Neisseria catarrhalis*, and *E. coli*.

The change in pH of the medium in which the control cultures were grown was determined with quinhydrone (Leeds and Northrup) and glass electrode (Coleman) potentiometers. Cultures other than the controls were not checked potentiometrically for pH changes. A qualitative pH trend was noted by observation of a color change in the indicator bromcresol purple.

RESULTS

Control cultures. The growth curve derived from multiplication of *T. foetus* in pure culture employing an inoculum of 50,000 organisms in 5 ml of medium was obtained from 2,487 counts on 178 cultures and can be noted in figures 1 to 6. An initial stationary phase and a lag phase were not evident. Apparently, the flagellate began logarithmic growth at the time of inoculation and continued in this phase for approximately 72 hours of incubation. The trichomonads multiplied at a decreased rate from 72 to 92 hours, and from 92 to 108 hours the flagellates had reached their maximum stationary growth phase. The peak of growth was attained in 100 hours, at which time the direct count was approximately 3.5 million organisms per ml. The period from 108 to 144 hours might be considered the phase of accelerated death, and the period from 144 to 216 hours the logarithmic death phase. After 216 hours, the numbers of trichomonads decreased very rapidly until none could be counted after 240 hours of incubation.

The initial pH of 7.2 was gradually reduced to a final pH of 5.18. The pH change was most rapid from 36 hours to 144 hours. After 144 hours the pH decreased from 5.4 to 5.18.

Corynebacteria. Figure 1 shows the effect of six species of corynebacteria on the development of *T. foetus in vitro*. Nine cultures of diphtheroids isolated from the genital tract of bulls were tested for their effect on the growth rate of this trichomonad, and the results are represented in figures 2 and 3.

The most striking point concerning figure 1 is the extreme prolongation of

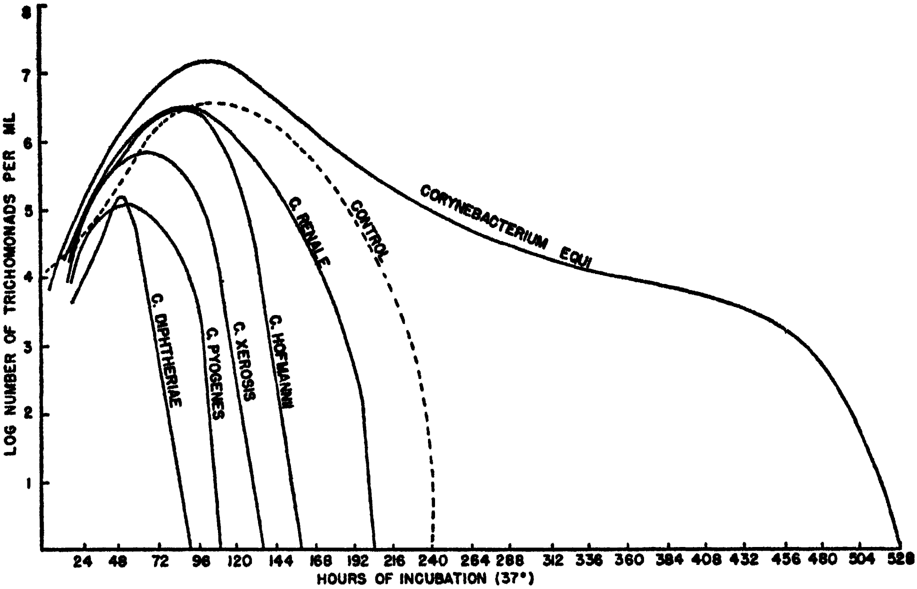

FIG. 1. GROWTH OF TRICHOMONAS FOETUS WITH VARIOUS SPECIES OF CORYNEBACTERIA

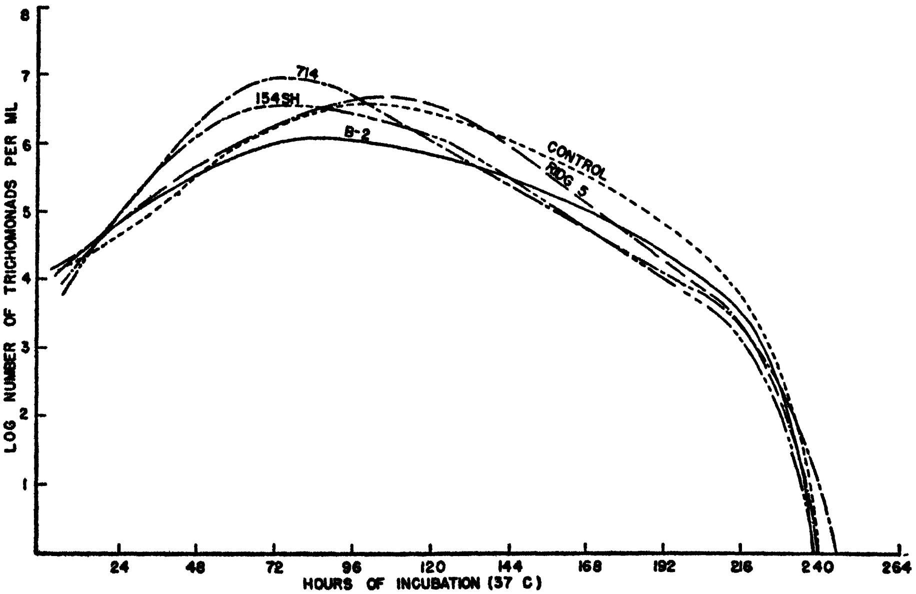

FIG. 2. GROWTH OF TRICHOMONAS FOETUS WITH VARIOUS DIPHTHEROIDS ISOLATED FROM THE GENITAL TRACT OF BULLS

life afforded *T. foetus* when grown in association with *C. equi*. This necessitated the use of a different horizontal scale from that used to construct the other five graphs. In the presence of *C. equi* the trichomonads reached their peak of growth in 100 hours, as did the control cultures, but this peak was approximately 14 million organisms per ml as compared to the peak of 3.5 million reached by the control. One of the former replicate cultures attained a peak of approximately 18 million per ml. The longevity of *T. foetus* was extended from 240 to 528 hours by *C. equi*. With *C. renale*, *C. hofmannii*, and *C. xerosis*, growth of the trichomonads was initially accelerated for but a brief time. The peak of growth of trichomonads was reached in 88 hours with *C. renale* and *C. hofmannii* and in 62 hours with *C. xerosis;* death occurred in 202, 160, and 136 hours, respectively. *C. pyogenes* and *C. diphtheriae* were decidedly inhibitive of trichomonad growth. Both moved the peak of trichomonad activity to the left at 48 hours, and the death of the trichomonads occurred in 110 hours with *C. pyogenes* and in 88 hours with *C. diphtheriae*. With the exception of *C. equi*, none of the species of *Corynebacterium* in figure 1 allowed *T. foetus* to attain a peak of growth as high as the control cultures.

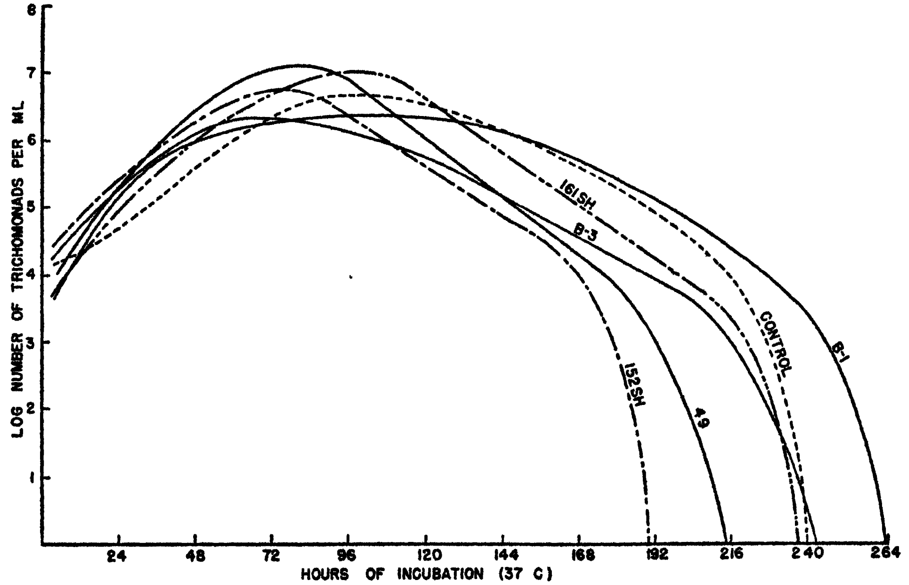

FIG. 3. GROWTH OF TRICHOMONAS FOETUS WITH VARIOUS DIPHTHEROIDS ISOLATED FROM THE GENITAL TRACT OF BULLS

In figures 2 and 3, diphtheroids are represented which were recovered in a previous study (Morgan *et al.*, 1946) from sheath swabs of clinically normal bulls with the exception of cultures B-1, B-2, and B-3, which were isolated from the seminal vesicle of a trichomonad-infected bull. Only two cultures, 152SH and 161SH, were identified; these were strains of *C. renale*. All of the diphtheroids accelerated multiplication of *T. foetus*, whereas 3 of them, B-1, B-2, and B-3, did not permit so high a peak of growth as was reached by the control. Most of the diphtheroids shifted the peak of trichomonad growth from 100 to 72 hours. One of the diphtheroids, B-1, was interesting in that it maintained the maximum stationary phase of trichomonad growth from 72 to 132 hours as compared to the range of 92 to 108 hours maintained by the control. Death of the flagellates occurred in 192, 216, and 264 hours with cultures 152SH, 49, and B-1, respectively. The other diphtheroids approximated the control in that activity of *T. foetus* subsided in about 240 hours.

Streptococci. Figure 4 indicates that in general the 10 streptococci studied are inhibitive to the multiplication of *T. foetus*. Only *S. bovis* (strain 5) gave any acceleration to the growth of the flagellates beyond that of the control growth curve, but this was short-lived with death resulting in 88 hours. *S. pyogenes* permitted longer survival of *T. foetus* than did other streptococci, but there was no acceleration of growth over that by the control. The peak of growth was not so high as that of the control, and death resulted in 202 hours.

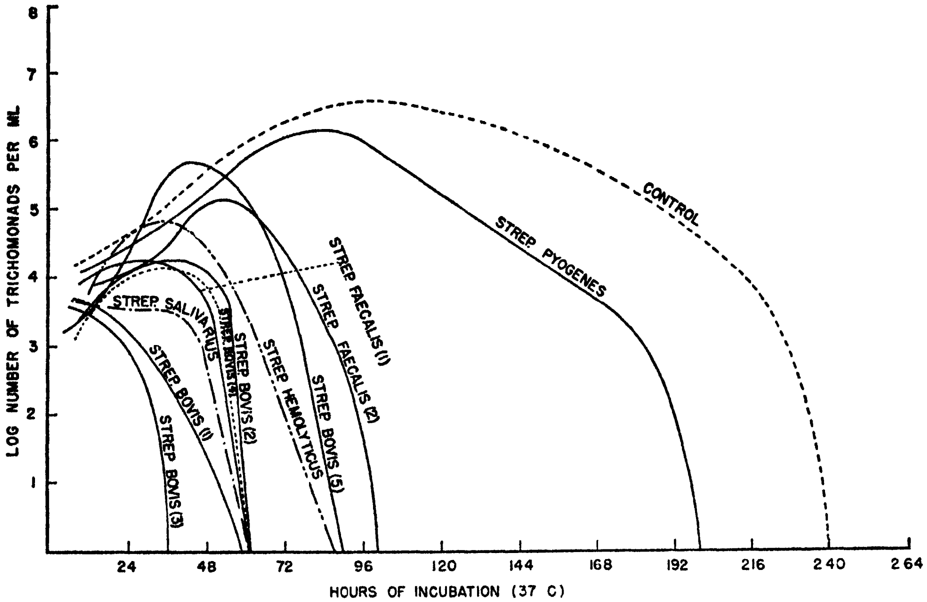

Fig. 4. Growth of Trichomonas foetus with Various Species of Streptococci

It is interesting to note the variation among the strains of streptococci. Strains 2 and 4 of *S. bovis* were very similar. Strain 3 caused death of the trichomonads in 36 hours, strains 1, 2, and 4 in 48 hours, and strain 5 in 88 hours. No acceleration of growth of *T. foetus* occurred with strains 1 and 3 of *S. bovis*, since the numbers of trichomonads decreased from the time of inoculation. Two strains of *S. faecalis* were inhibitory in their action on *T. foetus*. Strain 1 of *S. faecalis* produced death of the trichomonads in less than 62 hours and strain 2 in less than 88 hours, whereas Williams and Plastridge (1946), using a smaller inoculum, found that *T. foetus* did not survive so long as 24 hours with *S. faecalis*.

Miscellaneous bacteria. The results of tests on miscellaneous bacteria are shown in figures 5 and 6. Growth of trichomonads with *B. subtilis*, *P. aerugi-*

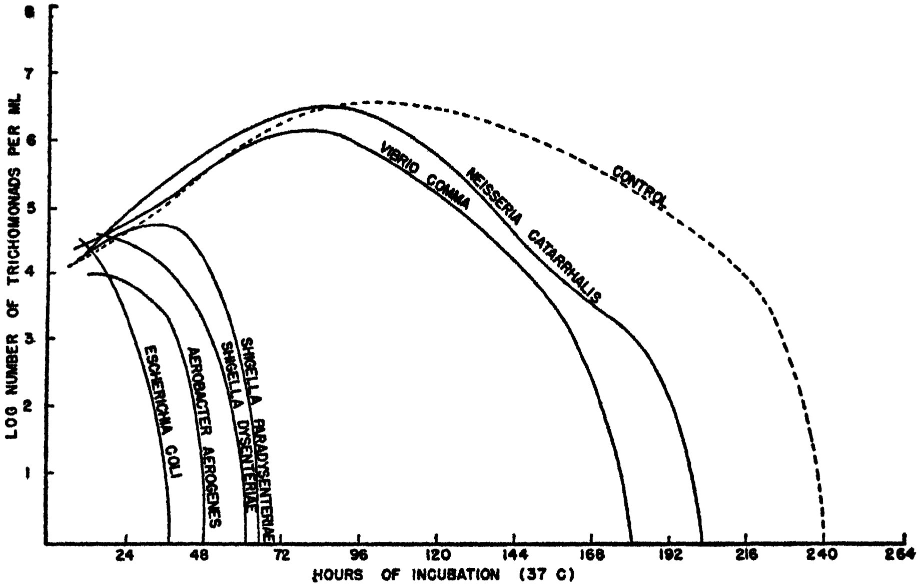

FIG. 5. GROWTH OF TRICHOMONAS FOETUS WITH VARIOUS SPECIES OF BACTERIA

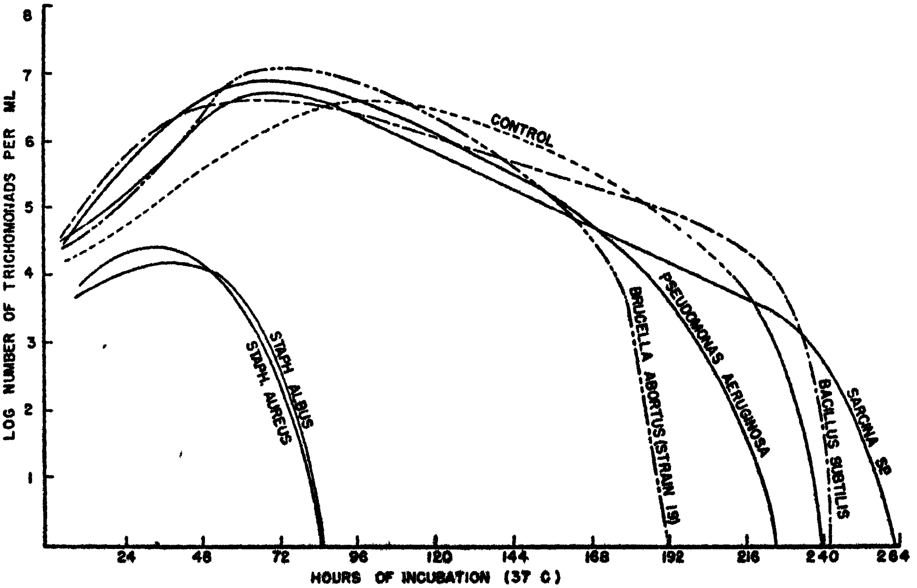

FIG. 6. GROWTH OF TRICHOMONAS FOETUS WITH VARIOUS SPECIES OF BACTERIA

nosa, *Brucella abortus* (strain 19), and *Sarcina* sp. was accelerated. *N. catarrhalis* slightly inhibited *T. foetus*. The peak of growth reached by *T. foetus* when growing with these cultures was equal to or, in most cases, greater than that of the control. Death of *T. foetus* occurred later (260 hours) than with the control only with *Sarcina* sp. Williams and Plastridge (1946) obtained somewhat different results with *P. aeruginosa.* They found that with a small inoculum *T. foetus* was killed within 48 hours when cultured with this bacterium.

The enteric group of bacteria, *E. coli*, *A. aerogenes*, *Shigella dysenteriae*, and *Shigella paradysenteriae*, and 2 species of staphylococci (*S. aureus* and *S. albus*) greatly inhibited the growth of *T. foetus.* The longevity of the trichomonads did not extend beyond 88 hours with any of these bacteria. *T. foetus* lived up to 48 hours with *E. coli* and *A. aerogenes*, whereas Williams and Plasteridge (1946), using a smaller inoculum, found these bacteria to kill trichomonads in less than 24 hours. These investigators also found *S. aureus* to kill trichomonads in less than 48 hours as compared to the 88-hour limit shown in these results.

Brucella abortus (strain 19) stimulated the growth of the trichomonads to as great a degree as any of the bacteria studied. When cultured in the presence of this bacterium, *T. foetus* reached a peak of over 11 million organisms per ml at 72 hours. However, the numbers of trichomonads declined rapidly thereafter until none could be found at 192 hours.

When grown with *V. comma*, the trichomonads showed a very slight initial growth acceleration over that of the control, but after 48 hours' incubation the growth curve stayed below the control, and the death of *T. foetus* occurred within 180 hours.

Growth of T. foetus with filtrates of bacterial cultures and heat-killed bacterial cultures. The development of *T. foetus* in association with various bacterial filtrates was essentially the same as that found with living bacteria. Trichomonad development in media containing heat-killed bacteria paralleled that already discussed for living bacteria and bacterial filtrates, with the exception of 3 species. With heat-killed *C. xerosis* cultures, growth was arrested some 20 hours later than in live cultures of this corynebacterium. Again, the maximum number of organisms did not reach that found in the bacteria-free control tubes. A slight prolongation of growth of *T. foetus* occurred in heat-killed cultures of *S. pyogenes* and *V. comma.*

DISCUSSION

The factors resulting in acceleration and inhibition of the growth of *T. foetus* with various bacterial cultures, filtrates, and heat-killed suspensions are not completely understood. A change in the hydrogen ion concentration of the medium may play a part, and, since the medium contains glucose, a higher acidity in general results from those bacteria capable of fermenting this monosaccharide than from those that do not. All of the bacteria that produced an inhibition of growth of the flagellate fermented glucose. However, some of the bacteria that accelerated the development of *T. foetus* also fermented glucose. *T. foetus* is capable of withstanding an extremely wide acid pH range (Morgan and Campbell, 1945), but, with certain rapidly growing bacteria, it may not be able to adjust itself to sudden pH reductions.

The exotoxin-producing bacteria were inhibitory to *T. foetus* without exception. The bacteria that killed trichomonads the quickest were not necessarily toxin producers since *A. aerogenes* and *E. coli* were more inhibitory than any bacterium studied with the exception of *S. bovis* (strain 3).

Since bacterial filtrates were comparable to living bacterial cultures in action upon *T. foetus*, it might be expected that the heat-killed toxin-producing species would, as a result of inactivating thermolabile toxins, affect the growth of the trichomonads in a different manner. However, there is some doubt that the heat treatment of 1 hour at 60 C was sufficient to destroy these toxins since, in most cases, there was such slight difference between the growth of *T. foetus* with living and heat-killed cultures of toxin-producing species. There appears to be little evidence from these results concerning the role of toxin production and its effect on the viability of *T. foetus*.

Some bacteria perhaps release valuable food products that would be unavailable to the trichomonads in pure culture. This may be the reason for the extreme prolongation of life of *T. foetus* by *C. equi* (figure1). The unusual action by *C. equi* may be attributed to the release of growth factors contained in the blood-egg portion of the modified Schneider's medium used in these studies. Unlike any of the other bacteria, *C. equi* caused an almost complete disintegration of the slant.

The diphtheroids (figures 2 and 3) were more consistently accelerative in their action upon *T. foetus* than any other group represented in this research. These organisms are common inhabitants of the uro-genital tract of cattle and are found frequently in close association with *T. foetus in vivo* (Morgan *et al.*, 1946). These organisms may play a part in the establishment of bovine genital trichomoniasis.

It seems that the action of various bacteria upon the growth of *T. foetus* involves three factors, namely, change in the hydrogen ion concentration, toxin production, and enzymatic release of growth substances in the medium which would be otherwise unavailable to the trichomonads.

SUMMARY

Certain bacteria have been found to accelerate and others to inhibit the growth *in vitro* of *Trichomonas foetus* as determined by direct microscopic counts of the trichomonads with a hemocytometer.

Bacteria that definitely inhibited the growth of *T. foetus* were *Corynebacterium diphtheriae*, *Corynebacterium pyogenes*, *Corynebacterium xerosis*, *Staphylococcus albus*, *Staphylococcus aureus*, *Streptococcus bovis* (five strains), *Streptococcus faecalis* (two strains), *Streptococcus salivarius*, *Streptococcus hemolyticus*, *Shigella dysentariae*, *Shigella paradysenteriae*, *Escherichia coli*, and *Aerobacter aerogenes*.

Those species that only slightly inhibited the growth of this flagellate were *Vibrio comma*, *Neisseria catarrhalis*, *Streptococcus pyogenes*, *Corynebacterium hofmannii*, and *Corynebacterium renale*. Bacteria that had no pronounced effect on the growth of *T. foetus* or which slightly accelerated the multiplication of this microorganism were *Bacillus subtilis*, *Pseudomonas aeruginose*, *Sarcina* sp., and nine cultures of diphtheroids isolated from the genital tract of clinically normal breeding bulls. *Brucella abortus* (strain 19) induced very rapid multiplication of the trichomonads but did not permit so long a survival time as the control cultures. One species, *Corynebacterium equi*, was outstanding in that it prolonged the life of *T. foetus* some 288 hours beyond the 240-hour limit of pure cultures of this trichomonad.

Bacterial filtrates and heat-killed cultures that were tested affected trichomonad growth in a manner comparable to the corresponding living bacteria cultured with *T. foetus*.

The probable nature of these accelerations and inhibitions has been discussed. It is believed that there are three main factors to be considered: (1) a change in the hydrogen ion concentration of the culture medium, (2) toxin production, and (3) release of additional nutrients favorable to trichomonad development.

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BIOLOGICAL STUDIES ON CAPSULATED YEASTS

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The phenomenon of extracellular starch production by capsulated yeasts has been briefly described in an earlier note (Aschner, Mager, and Leibowitz, 1945). In the present communication biological aspects of the production of starchlike substances and of a soluble capsule substance by yeast are investigated.

MATERIALS AND METHOD

The taxonomic nomenclature of Stelling-Dekker (1931) and Lodder (1934) is used throughout the paper. The yeast strains were derived partly from the stock of the Department of Bacteriology and Hygiene of the Hebrew University and partly from the following type culture collections: National Type Culture Collection, London; American Type Culture Collection, Washington; and the Centraalbureau voor Schimmelcultures, Yeast Division, Delft.[1] Several yeast strains were kindly provided by Dr. R. Benham, Columbia University, New York.

Nitrogen and carbon utilization was examined by the auxanographic method of Beijerinck (1889). An auxanographic procedure was also used to determine the vitamin requirements. Drops of solutions of vitamins were placed by means of a bacteriological loop on the surface of a solid medium containing KH_2PO_4, 0.1 per cent; $MgSO_4$, 0.05 per cent; glucose, 1 per cent; and $(NH_4)_2SO_4$, 0.1 per cent or its equivalent of norit-adsorbed acid hydrolyzate of casein. Circumscribed growth around the spot of deposition of the vitamin drop indicates a positive reponse to the vitamin. The diameter of the growth field is roughly proportional to the concentration of the vitamin in the drop (figure 1).[2] In some instances nitrate assimilation was examined by growing the yeast on a medium composed as described above but with 0.1 per cent KNO_3 as the sole source of nitrogen. After a growth period of 2 to 4 days the Griess-Ilosway reagent for nitrite was poured on the culture plate. Good growth on nitrate medium was always accompanied by a positive nitrite reaction.

CONDITIONS OF STARCH PRODUCTION

Most of the work on the conditions governing the appearance of starch (or starchlike substance) was done with a strain isolated from the air at Jerusalem and identified as *Torulopsis rotundata.*

The yeast was inoculated into various nutrient media and the appearance of starch or starchlike material was determined by the addition of Lugol's iodine solution. Starch failed to appear when the yeast was cultivated on peptone glucose agar, although this medium supported a luxuriant growth. On a peptone-free medium containing ammonium sulfate as the source of nitrogen and glucose as the source of carbon, the growth of the yeast was very poor and there was no starch production. When, however, the same medium was enriched by the addition of a small quantity of yeast or meat extract, normal growth with abundant formation of starch was achieved. Other ammonium salts such as ammonium chloride or ammonium phosphate maintained an equally rich growth and good starch production. When the ammonium salts were replaced by a nitrate salt, such as potassium nitrate, and supplemented with yeast extract, the growth was even more luxuriant but no starch could be detected in the culture. With ammonium nitrate good starch formation was obtained, but in this case ammonium and not nitrate was the chief ion assimilated.

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[1] We are indebted to Dr. J. Lodder for valuable suggestions in the choice of strains.

[2] The authors are indebted to Miss L. Bichowsky for her collaboration in the development of this method.

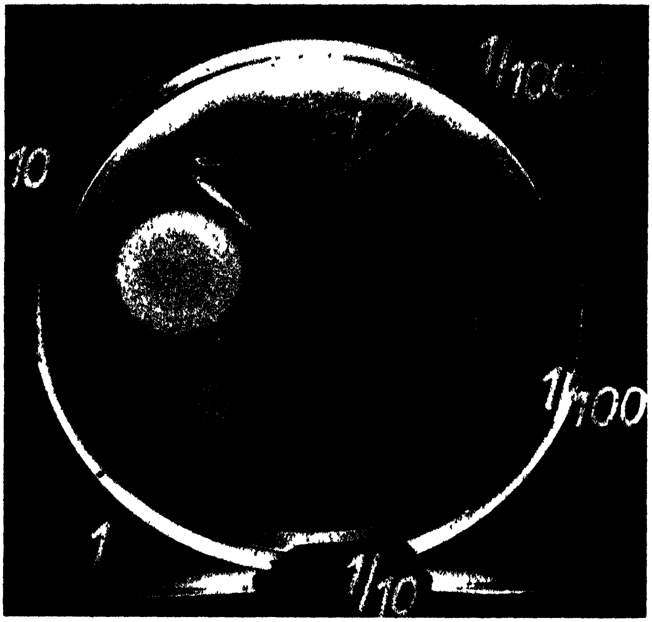


FIG. 1. A VITAMIN AUXANOGRAM

Growth response of *Rhodotorula* sp. to different concentrations of thiamine (0.001 to 10 micrograms per 1 ml).

In the light of these results it seemed probable that the production of starch is governed, under conditions for good growth, by the source of nitrogen supplied. Subsequently, however, it was found that the primary essential condition for the production of starch is a shift of the pH of the medium during growth to a value below pH 5. When *T. rotundata* grows on ammonium sulfate, it assimilates ammonium cation and releases sulfate anion. Thus, a shift of pH from 6 to 2 is produced, at which point growth itself is checked. If acidification is prevented by the addition of calcium carbonate, the production of starch is suppressed.

When either peptone or nitrate salts or casein hydrolyzate served as a source of nitrogen, acidification did not occur and starch therefore was not formed. On the other hand, when the yeast was cultivated on a medium containing casein hydrolyzate that was buffered at pH 3 to 4 with McIlvain buffer solution, starch production could be demonstrated. The starch reaction was weak because the buffering power was insufficient to maintain a constant pH below 4, and soon a shift into the alkaline range occurred.

EXPERIMENT WITH RESTING CELLS

It could be argued that the seeming dependence of extracellular starch formation on growth is only secondary and a result of acidification of the medium in the course of anabolic processes. In order to clear up this question the following experiment was made:

Yeast cells harvested from peptone agar cultures (in which no starch production occurs) and thoroughly washed with distilled water were added in thick suspension to a series of tubes containing 1 per cent glucose, thiamine, and McIlvain's citric acid, phosphate buffer. The buffer solutions ranged in steps of 0.5 from pH 3.6 to pH 6. Daily small samples were taken and tested with Lugol's iodine solution for starch. No starch production was observed even after an incubation period of 2 weeks.

The negative outcome of the resting cells experiment demonstrates clearly that the phenomenon of starch production is intimately bound to active cell proliferation. Starch production by *T. rotundata* depends on good growth and the concurrent lowering of the pH to below 5.

PRODUCTION OF STARCH IN SYNTHETIC CULTURE MEDIA

To obtain information concerning the accessory factors needed by *T. rotundata* we made up a synthetic medium composed as follows: $(NH_4)_2SO_4$, 0.1 per cent; KH_2PO_4, 0.1 per cent; $MgSO_4$, 0.05 per cent; glucose, 1 per cent; and agar, 2.5 per cent. On this basal medium the following mixture of vitamins was tested by the auxanographic method:

Riboflavin	20	Calcium pantothenate	20
Nicotinic acid	25	Pyridoxine hydrochloride	20
m-Inositol	100	Cholin chloride	100
Pimelic acid	10	Biotin	5
p-Aminobenzoic acid	20	Thiamine hydrochloride	20

The concentrations are given as micrograms per ml.

The growth of *T. rotundata* on this medium was as good as that on a similar medium containing, instead of vitamin mixture, a yeast or meat extract or tomato juice. The production of starch on the synthetic medium was even more profuse than in the extract-containing media.

By a procedure of elimination it was found that the only vitamin required for good growth is thiamine. A concentration of 0.2 μg per ml was enough to support optimum growth after 2 days' incubation at 30 C.

In a liquid medium of the same nutrient composition the growth was very slow and starch made its appearance only after a period of about 2 weeks' incubation. As *T. rotundata* is a nonfermenting yeast with a pronounced oxidative metabolism, it was reasonable to assume that the limiting factor in the liquid medium is the supply of oxygen. This assumption was verified by a growth experiment in which the same liquid medium was used but vigorous aeration by constant shaking or bubbling of air was applied. Under these conditions growth was again rapid and profuse, and the starch reaction was positive at the end of 1 to 2 days of growth.

MORPHOLOGICAL OBSERVATIONS ON THE APPEARANCE OF STARCH IN CULTURES OF T. ROTUNDATA

Cells of *T. rotundata* from 3- to 5-day cultures on peptone glucose agar appeared in the microscopic field either round or oval, with regular edges and conspicuous capsules. When ammonium sulfate agar cultures were examined microscopically after the same period of growth, they presented an entirely different picture. Most of the cells were distended and of irregular outlines, sometimes almost ameboid. The capsule was partly detached from the cell wall and the protoplasm shrunken. In many cells the capsule was ruptured at one point and seemed as if about to be shed. With the addition of a drop of Lugol's iodine solution, the starch or starchlike material could be seen accumulated in the capsule or in the form of very small granules outside the cells. No traces of starch but inclusions staining brown with iodine, as does glycogen, were visible within the protoplast. Some starch was obviously also dissolved in the culture medium as is shown by the fact that the cell-free supernatant gave an intense blue coloration with iodine after centrifugation.

SPECIFICITY OF SUBSTRATE

The dependence of starch elaboration on the structure of the carbon source was tested in the medium described above by the addition of various organic compounds in the place of glucose (table 1).

As may be concluded from table 1, compounds which permit growth and acidification of the culture medium serve also as substrates for starch production. It is interesting to note that, as in the case of *Saccharomyces*, none of the phosphate esters which are intermediaries of glycolysis is assimilated. In addition, a strain of the pathogenic *Torulopsis neoformans* was examined as to its ability to utilize the various carbon sources as substrates for starch production. Essentially the same results were obtained as with *T. rotundata.*

INFLUENCE OF SUGAR CONCENTRATION ON GROWTH AND STARCH PRODUCTION BY T. ROTUNDATA

Since we intended to isolate the starch from the culture medium for a study of its chemical properties, it appeared important to study the influence of sugar concentration on the starch yield. This was done by cultivating the yeast in constantly shaken liquid media containing increasing amounts of glucose. After 48 hours of incubation the amount of growth and of extracellular starch produced in the various sugar concentrations was determined photometrically. The concentration of starch in the medium was determined by measuring the extinction value of the blue color obtained by the addition of 0.1 ml iodine solution (N/100) to a 5-ml sample of cell-free culture medium. At the same time colorimetric determinations of pH were made in the cultures.

TABLE 1

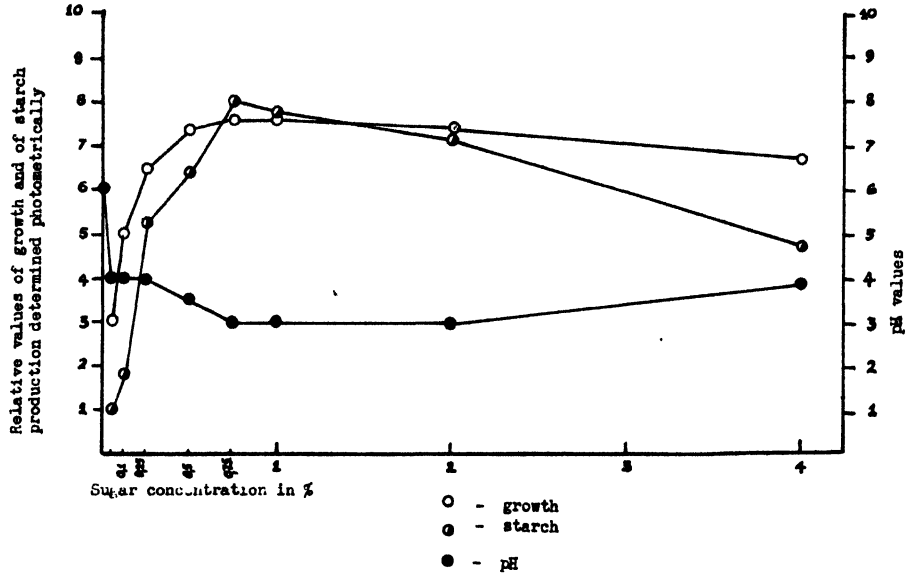
Suitability of various carbon sources for growth and starch production

CARBON SOURCE	GROWTH	STARCH PRODUCTION
Glucose	+	+
Mannose	+	+
Galactose	–	–
Fructose	+	+
Sorbose	–	–
d-Xylose	+	+
l-Arabinose	+	+
d-Arabinose	–	–
d Lyxose	–	–
d-Ribose		–
l-Rhamnose	+	+
Mannitol	+	+
Sorbitol	+	+
Adonitol	–	–
Erythritol	–	–
Glycerol	±	±
Inositol	+	+
Lactose	–	–
Sucrose	+	+
Maltose	+	+
Methyl α glucoside	+	+
Cellobiose	+	+
Melibiose	+	–
Trehalose	–	–
Raffinose	±	±
Glycogen	–	–
Soluble starch	–	–
Levan	–	–
Dextran	–	–
Lichenin	–	–
Inulin	–	–
Cori ester	–	–
Neuberg ester	–	–
Hexose diphosphate	–	–
Calcium gluconate	+	–
Peptone (sole source of C and N)	±	–

Graph 1 shows that the optimum of growth is attained at a sugar concentration of 0.5 to 0.8 per cent. The starch production curve runs parallel to the growth curve but shows in the range of inhibitory glucose concentrations a steeper decline. The optimum peak is therefore sharper. The lowest pH value coincides with *the optimum of growth and starch production.* In this case about 1 per cent of the glucose is converted to starch (graph 1).

BIOLOGICAL SPECIFICITY OF THE PHENOMENON OF STARCH FORMATION

After conditions which are necessary for the elaboration of extracellular starch by *T. rotundata* had been established, we examined a large number of yeast species, representing widely different taxonomic groups, for the ability to produce starch or starchlike substances. The tests were performed on the solid medium already described by seeding the yeasts on sectors of the agar plate and testing the agar beneath the growth streak with iodine for starch. Instead of thiamine only the mixture of vitamins was added to the culture medium. Under these conditions all our strains, even those described in the literature as incapable of utilizing $(NH_4)_2SO_4$, e.g., *Torulopsis molischiana,* proved capable of cultivation of ammonium sulfate agar. As may be seen in table 2, ability to produce starch is restricted exclusively to capsulated nonfermenting and nonsporulating yeasts, including pathogenic as well as nonpathogenic species.



GRAPH 1. INFLUENCE OF SUGAR CONCENTRATION ON GROWTH, pH, AND STARCH PRODUCTION IN CULTURES OF T. ROTUNDATA

In the photometric values given, 10 denotes total extinction. Determinations were made after 48 hours of incubation at 30 C.

Minor differences in the amount of starch produced and in the rapidity of its appearance could be observed. In some strains the starch reaction was perceptible only after a week's incubation. In general, starch production seemed to be correlated with the ease of assimilation of $(NH_4)_2SO_4$ and with the rate of the acidification of the medium.

PRESENCE IN CULTURES OF CAPSULATED YEASTS OF A SOLUBLE POLYSACCHARIDE OTHER THAN STARCH

In our preliminary note we mentioned that extracellular starch appears always in association with another polysaccharidic component which exhibits properties entirely different from the starch fraction. A polysaccharidic material which does not stain blue with iodine has been separated by alcohol precipitation from cultures of capsulated yeasts grown under conditions in which starch production was prevented by the use of nitrogen sources which do not allow acidification of the medium (e.g., potassium nitrate, casein hydrolyzate, or urea). This soluble material was produced from all the carbon sources which maintained growth. In a chemical investigation which will be recorded elsewhere, it was revealed that the nonstaining substances of *T. rotundata* and *T. neoformans* are largely carbohydrates. The carbohydrate fraction is made up of pentose (85 per cent) and hexose (15 per cent). The substance is obviously identical with the pentose-containing material that appears in ammonium sulfate medium in association

TABLE 2

Starch production by various yeasts

STARCH-NEGATIVE STRAINS	STARCH-POSITIVE STRAINS
Candida albicans	*Torulopsis albida*
Debaryomyces kloeckeri (2 strains)	*Torulopsis diffluens*
Debaryomyces matruchoti (4 strains)	*Torulopsis flavescens*
Geotrichum dermatitidis (2 strains)	*Torulopsis innocua*
Hansenula sp.	*Torulopsis laurentii*
Kloeckera sp	*Torulopsis liquefaciens*
Mycoderma sp	*Torulopsis luteola*
Mycotorula sp. (3 strains)	*Torulopsis mucorugosa*
Pichia sp.	*Torulopsis nadaensis*
Pityrosporum ovale	*Torulopsis neoformans* (16 strains)
Rhodotorula mucilaginosa	*Torulopsis rotundata* (2 strains)
Rhodotorula bronchialis	*Candida** *heveanensis*
Saccharomyces cerevisiae	
Saccharomyces ellipsoideus (3 strains)	
Saccharomyces carlsbergensis	
Schizosaccharomyces sp. (2 strains)	
Sporobolomyces sp.	
Torulopsis candida	
Torulopsis colliculosa (2 strains)	
Torulopsis glabrata	
Torulopsis minor	
Torulopsis molischiana	
Torulopsis utilis (2 strains)	
Torulopsis sp. lactose-fermenting (2 strains)	
Zygosaccharomyces sp	

* We believe that *C. heveanensis* is closer to *Torulopsis* than to *Candida* and should be renamed *Torulopsis heveanensis*.

with starch. A positive reaction for pentose was also noted in preparations of the soluble polysaccharidic substance isolated by alcohol precipitation from cultures of other capsulated strains: *Torulopsis neoformans* (4 strains), *Torulopsis mucorugosa*, *Torulopsis innocua*, and *Torulopsis liquefaciens*. Under the same growth conditions no extracellular substance could be isolated from cultures of *Saccharomyces cerevisiae*, *Torulopsis utilis*, or *Torulopsis glabrata*, nor could pentose be demonstrated in their culture fluids.

DISSOCIATION IN T. ROTUNDATA: CAPSULAR ORIGIN OF THE SOLUBLE SUBSTANCE

From the fact that the pentose-containing soluble substance was only found in cultures of capsulated yeasts it seemed to be likely that the substance is derived from the capsule. This assumption gained strength by the finding that 1 to 2 mg of washed dried cells of capsulated yeasts afford a rapid and intense reaction for pentose with Bial's reagent. Cells of noncapsulated yeasts also give a positive pentose reaction, presumably because of their nucleic acid content, but only if a much larger quantity is used.

In the absence of a suitable cytochemical reaction, direct evidence in favor of the foregoing hypothesis could not be obtained. We attempted therefore to isolate capsule-free variants of the yeasts in order to ascertain whether such variants are able to produce pentose-containing soluble substance. The absence of this ability in a variant devoid of the capsule structure would of course lend support to the hypothesis that this soluble substance is of capsular origin.

Attempts to induce an S to R variation by procedures that Fabian and McCullough (1934) successfully applied to other yeast species proved unsuccessful. Incidently, however, an observation was made which enabled us to achieve our objective. While examining the ability of *T. rotundata* to utilize lactose, we observed an auxanogram response peculiarly different from that shown to other sugars. During an incubation period of about 2 weeks no response at all could be detected. Then a few small colonies began to appear in the diffusion field of lactose. The number of the colonies was much below the number of cells originally seeded in the agar. When subculture of this growth on glucose peptone agar was attempted, two colony types grew out: (a) large, flat, rapidly growing, grayish and mucoid colonies; and (b) small, convex, slow growing, dry-looking colonies that tended to turn brown after 10 to 14 days. In accordance with the nomenclature adopted by Dawson (1934) for pneumococci variations, the first type, which looked in all respects identical with the original form, was termed the M form and the second type, the S form. On subculturing colonies from the S phase, colonies with pronounced "rough" characteristics were obtained. The R forms were, however, unstable and tended to reverse to the usual S form. When examined microscopically (figures 2 and 3), the M form cells presented a uniform picture of round or slightly oval cells with three zones: a vacuolated protoplast, the cell wall, and a broad capsule. The S form presented a quite different appearance. Most of the S form cells were devoid of a capsule; frequently elongated cell groups were encountered; some times, fairly well-developed pseudomycelia could be found.

Biochemically, the two forms differ only by the fact that whereas the S form grows normally on lactose and galactose as sole sources of carbon, growth of the M form on these sugars is very poor. The two forms remained true to type after 1½ years of cultivation on our usual glucose stock medium. The S form could be obtained from the M form repeatedly by the technique described. The reverse change from S to M has not been observed. Thus, the lactose-utilizing type appears to be the stable form, whereas the lactose-negative type is able to split off lactose-positive variants continuously. This behavior recalls the variation pattern of *Escherichia coli-mutabile*. The essential identity of the two forms of *T. rotundata* was further confirmed by the fact that both of them produced starch under the proper conditions. However, the starch produced by the S form is found only in the cell walls but not in the cell-free supernatant of the culture. The chief point of difference is that no pentose

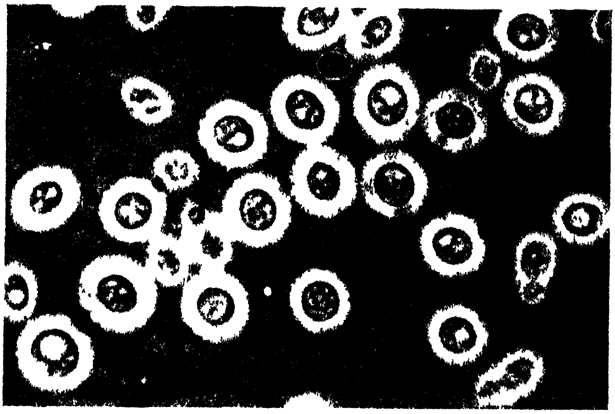


FIG. 2 CELLS OF T. ROTUNDATA FROM AN M COLONY ×750

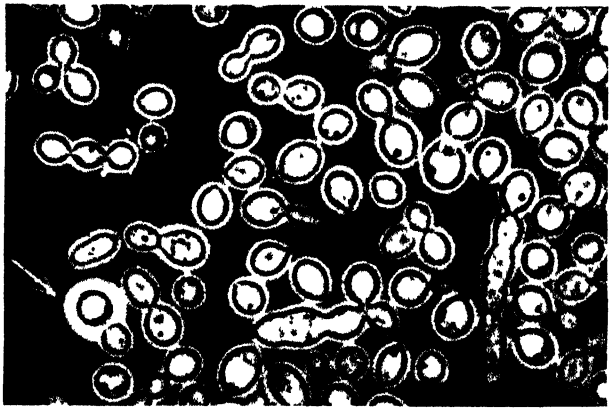


FIG. 3 T. ROTUNDATA IN S PHASE

Only one cell shows capsule formation. Note elongated cell groups. ×750 can be demonstrated in the supernatant of S form cultures, nor can polysaccharidic substance be precipitated from the medium by alcohol. The few remaining capsulated cells in the S form are obviously insufficient to produce a perceptible quantity of the soluble substance. This finding is consistent with the fact that the S form can be readily sedimented, whereas the M form fails to form a packed sediment even after very prolonged centrifugation. The results, therefore, confirm the view that the soluble polysaccharidic material which does not stain with iodine derives from the capsule.

IMMUNOLOGICAL PROPERTIES OF THE CAPSULAR MATERIAL OF T. ROTUNDATA

In order to test the serological qualities of the capsular material, two rabbits were immunized with heat-killed yeast cells, and two rabbits with living cells.

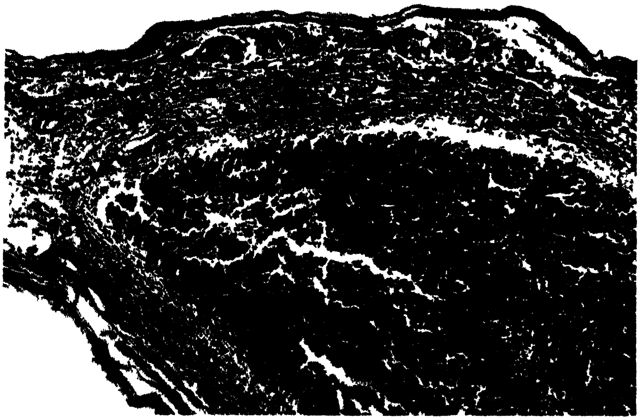


FIG. 4. ABSCESS CAUSED BY T. ROTUNDATA ON EAR OF A RABBIT

Sections stained with hematoxylin-eosin ×30

The injections were made initially subcutaneously, and later intraperitoneally and intravenously at time intervals of 5 to 6 days. The injections were continued for a period of 4 months, and the dose was successively increased until a whole agar slant culture was given in a single intravenous injection. In spite of this intensive treatment, no antibodies, neither agglutinins against the cells nor precipitins against the capsular material, could be detected at the end of the period. Similar results were obtained when the cells were first subjected to a mild acid digestion in the cold, as advised by Benham (1935). With this method Benham obtained low titer antisera against several capsulated strains.

In another experiment, three guinea pigs were given intraperitoneal injections of dead and living yeast cells for a period of 3 weeks, at intervals of 2 to 3 days. After a rest period of 21 days an attempt was made to elicit anaphylactic shock by injecting intracardially either dead cells or 100 mg of capsular substance. No signs suggestive of an anaphylactic reaction were found.

A cutaneous reaction could be demonstrated in rabbits which had developed abscesses in the ear skin following repeated massive injections of living cells into the ear: when a suspension of dead yeast cells was injected into the abdominal skin of such rabbits erythema (3 cm in diameter) appeared at the site of the injection and lasted for about 24 hours. In healthy rabbits no skin reaction was produced by the injection of dead cells.

INOCULATION EXPERIMENTS WITH T. ROTUNDATA ON RABBITS

In the usual animal tests *T. rotundata* proved to be nonpathogenic. When the yeast was injected under or into the skin of the abdomen, abscesses appeared in a few cases but healed rapidly. On the other hand, abscesses produced on the ear skin persisted for months. Histological preparations showed marked infiltrations with mononuclear elements and scattered yeast cells (figures 4 and 5). The yeast could still be cultured from such lesions several months after the last injection. This peculiar behavior is understandable in view of the inability of *T. rotundata* to grow at temperatures near 37 C. It is suggested that the yeast is able to maintain its viability for a long period of time in the ear skin, and to produce pathological alterations in this site, because the temperature of the ear skin of the rabbit is less than that of other parts of the body.

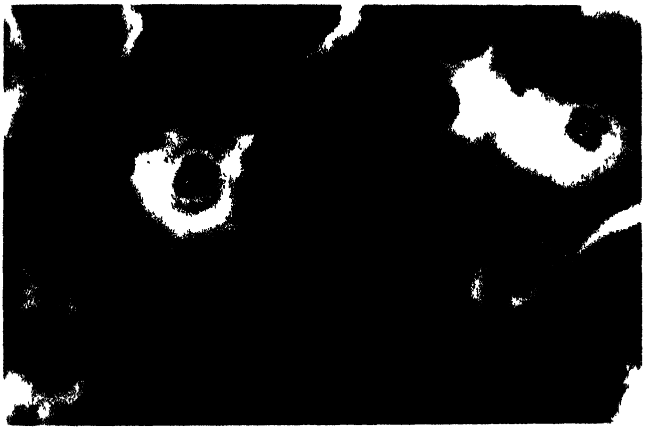


FIG. 5. YEAST CELLS IN TISSUE (DETAIL FROM PREPARATION OF FIGURE 4) ×1,000

DISCUSSION

Polysaccharides which stain blue with iodine are rare outside the realm of green plants. One of the well-known examples is the so-called granulose or iogen of certain clostridia of the butyricum group (Trécul, 1865). Although accurate data concerning the chemical composition of this substance are not available, it seems probable that it corresponds to a starch fraction and serves as a kind of reserve material. This may be true also of the role of the starch material detected by Beijerinck (1897) in ascospores of *Schizosaccharomyces*. In both of these instances the starchlike material is bound to the cells and is never found extracellularly in the culture medium. The phenomenon we have described differs from the cases cited in two important points: (a) the starch accumulates only extraplasmatically, i.e., in the cell wall or in the capsule and in the culture medium outside the cells; and (b) the elaboration of the starch is limited to growth conditions which ensure production of an acid pH. In both these respects the starch of capsulated yeasts resembles the mold starch described for some species of fungi by Boas (1917) and Schmidt (1925). From the ability of the yeasts to convert into starch organic compounds that differ widely from glucose in their chemical structure, it can be inferred that the production of starch is in some way linked to vital metabolic processes. This conclusion is further supported by our finding that starch production by living yeasts depends on active cell proliferation. The association of the phenomenon with abnormal conditions of growth and the microscopic appearance of the starch-containing cells suggest that the appearance of starch in the medium of capsulated yeasts represents a pathological deviation of the metabolism. The fact that this property has been found to be an exclusive characteristic of a group of capsulated yeasts suggests that there may be some kind of interrelationship between the capsule structure and the starch production. The capsule material of all nonfermenting strains so far tested contains pentose. This finding, like other biochemical and morphological features common to these yeasts, points to the view that there is a close phylogenetical relationship between these yeasts.

The capsular substance of *T. rotundata* in contrast to most bacterial capsular substances has been proved to be entirely devoid of immunological activity. This is in agreement with the suggestion of Benham (1935) that the capsular material is responsible for the immunological inertness of capsulated yeasts. It may also be that this substance is one of the determining factors of the chronic and usually fatal course of infection with *T. neoformans*.

It appears that the pathogenicity of various species of capsulated yeasts is a function of their optimum growth temperature (Benham,1935; Mager and Aschner, 1946). This assumption is supported by the results of our animal experiments with *T. rotundata*. The theory can be put forward that pathogenic yeast strains are derived evolutionally from nonpathogenic ones by a mutation of a factor which determines temperature tolerance.

SUMMARY

The conditions governing the elaboration of extracellular starch by capsulated yeasts were determined. Thirty-seven different yeast species belonging to 15 genera were tested for the ability to produce extracellular starch. It was found that this ability is restricted to capsulated nonfermenting asporogenous yeasts.

The growth medium of capsulated yeasts also contains, apart from starch, a soluble polysaccharide that does not stain blue with iodine and consists largely of pentose. Evidence is adduced in support of the view that this substance derives from the capsule.

A dissociation phenomenon in *Torulopsis rotundata* was noted.

Immunization attempts with the capsular substance of *T. rotundata* gave negative results.

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STUDIES ON NONFRUITING MYXOBACTERIA

I. CYTOPHAGA JOHNSONAE, N. SP., A CHITIN-DECOMPOSING MYXOBACTERIUM

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The concept of myxobacteria as a microbial assemblage characterized by a complex life cycle involving a unicellular phase of vegetative proliferation and a communal phase of fructification was clearly expressed by Thaxter (1892) in the first detailed publication dealing with the group. This concept remained unchallenged for forty years. Only during the past decade has the realization gradually arisen that "imperfect myxobacteria," to use the phrase of Imsenecki and Solntzeva (1945), which lack a fructificative stage but are recognizable by other means as members of the group, may also exist. The best-known nonfruiting myxobacteria are the cellulose-decomposing soil cytophagas, whose true systematic position, long obscure, has been gradually revealed by the discovery and recognition of their numerous myxobacterial properties: microcyst formation in some species (Krzemieniewska, 1930; Imsenecki and Solntzeva, 1936), creeping motility and flexing movements of the individual cells (Stapp and Bortels, 1934), and swarming on solid media (Stanier, 1940). The classical cellulose-decomposing soil cytophagas have a high degree of nutritional specialization, and although it has recently been discovered that some simpler carbohydrates can be metabolized in addition to cellulose (Fåhraeus, 1941; Stanier, 1942), they remain a sharply delimited group from the nutritional standpoint. However, a considerable number of nonfruiting myxobacteria with much broader nutrient requirements are now known. The first to be described were the marine agar-decomposing cytophagas, which, in contrast to the then known soil forms, could use peptides and a wide range of carbohydrates as energy sources, were not inhibited by heat-sterilized sugars, and required growth factors for their development (Stanier, 1940). During the past five years a considerable number of nonfruiting myxobacteria with varied nutrient requirements and morphological properties have been described by Fuller and Norman (1943), Imsenecki and Solntzeva (1945), Soriano (1945), Harmsen (1946), and Humm (1946). As Imsenecki and Solntzeva have pointed out, "imperfect" myxobacteria are not uncommon in nature, although for various reasons their recognition may at times be difficult.

A few years before the existence of nonfruiting myxobacteria was generally recognized, Johnson (1932) published a brief account of certain myxobacteria which attack chitin. Some of these organisms appear to have been typical *Myxococcus* spp., but others failed to form fruiting bodies while possessing the vegetative morphological characteristics of the fruiting types. Johnson's de-

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[1] Fellow of the John Simon Guggenheim Memorial Foundation, 1945–1946.

scriptions, although they suggested that these forms might be nonfruiting myxobacteria of the *Sporocytophaga* and *Cytophaga* types, were not complete enough to permit a final decision without further investigation. The present work was accordingly started in an attempt to confirm and extend her findings.

MATERIALS

Chitin was prepared from lobster shells. After decalcification with dilute HCl, the shells were boiled for 12 hours in 10 per cent KOH, washed, treated with dilute $KMnO_4$ for 20 minutes at 60 C, washed again, suspended in a cold concentrated solution of sodium bisulfite until decolorized, and dried at 80 C, after a final thorough washing to remove the last traces of bisulfite. The leathery strips of material thus produced were used without further treatment in enrichment cultures. For incorporation in agar media, a very finely divided suspension of chitin was prepared by dissolving strips purified as above in cold 50 per cent H_2SO_4 and reprecipitating by rapid dilution with a 10- to 20-fold volume of water. The resulting product was freed from acid by repeated centrifugation and washing, and kept as a sterile aqueous suspension. Material prepared in this way is somewhat gelatinous and cannot be dried successfully, as it coheres during drying into a hard, horny mass impossible subsequently to resuspend.

Enrichment cultures for chitin-decomposing bacteria were set up by adding strips of chitin to shallow layers of a dilute mineral base (K_2HPO_4 0.1 per cent, $MgSO_4$ 0.05 per cent, NaCl 0.05 per cent, $CaCl_2$ 0.01 per cent, $FeCl_3$ 0.005 per cent, pH 7.0 to 7.2) in Erlenmeyer flasks, which were inoculated with soil or mud and incubated at 25 to 30 C. Some anaerobic enrichment cultures were also set up, using the same medium in completely filled glass-stoppered bottles, but no chitin decomposition was observed in them.

Mineral chitin agar was prepared by the addition of agar and chitin suspension to the foregoing mineral base in the requisite amounts. The same mineral base with 0.1 per cent NH_4Cl was employed for testing the utilization of carbon compounds, which were added in a concentration of 1.0 per cent. Utilization was determined by growth in comparison with that in an inoculated control not containing any carbon source. The utilization of nitrate as a hydrogen acceptor was tested by cultivation in glass-stoppered bottles filled with a medium consisting of 0.5 per cent tryptone and 0.5 per cent KNO_3. All other media were prepared in the customary manner.

ENRICHMENT AND ISOLATION OF CHITIN-DECOMPOSING MYXOBACTERIA

In aerobic enrichment cultures prepared as described above, the chitin undergoes slow decomposition at 25 to 30 C, accompanied by the development of an extremely varied microflora consisting only in part of chitin-decomposing microorganisms. Streaks from such enrichment cultures on 2 per cent agar plates containing the above-listed minerals, together with a finely divided suspension of chitin, usually reveal several eubacterial types as the most conspicuous chitin decomposers; they are for the most part pseudomonads, which produce small white or pale yellow colonies surrounded by cleared zones in which the chitin has been dissolved. Occasionally chitin-decomposing *Streptomyces* spp. will be found. However, examination of such plates after 5 to 6 days also almost invariably reveals the presence of chitin-decomposing myxobacteria, although from their inconspicuous manner of growth they can easily be overlooked on cursory examination. Sometimes they are the only chitin decomposers to develop in an enrichment culture. Their colonies consist of thin, translucent, pale yellow, almost colorless swarms, which spread with great rapidity and soon cover large areas of the plate with a thin layer of cells. Because of their thinness, young swarms may be invisible by transmitted light, but when examined by reflected light their matt surface shows up in contrast to the surrounding agar. Except on old plates chitin decomposition is never very conspicuous, since the partial clearing which occurs beneath the swarm is masked by the overlying layer of cells, and because of the rapid extension of the swarm there is usually not a cleared zone beyond its periphery.

A number of strains were picked and purified for further study. At first, purification was attempted by cutting out small sections of agar at the periphery of the swarm and transferring them to fresh chitin agar plates; but after the discovery that abundant growth occurred on peptone or yeast extract agar, more expeditious and certain purification was achieved by streaking on plates of these media in the customary manner.

PROPERTIES OF THE ISOLATED STRAINS

All the strains isolated in the manner just described have proved to be nonfruiting and amicrocystogenous, thus conforming to the definition of the genus *Cytophaga*. Microcystogenous and fruiting myxobacteria were never encountered during this work, either in enrichment cultures or on plates, although the observations of Johnson (1932) suggest that chitin decomposers with these morphological properties also exist. The provenance of the strains studied in detail is shown below.

Strain 1. From enrichment culture inoculated with mud; Cambridge.
Strain 2. From enrichment culture inoculated with garden soil; Rothamsted.
Strain 3. Same source as 2.
Strain 4. From enrichment culture inoculated with field soil; Rothamsted.
Strain 5. Same source as strain 4.
Strain 6. From enrichment culture inoculated with compost; Rothamsted.

Morphology. The vegetative structures are thin, weakly refractile rods of even width, with rounded ends. In young cultures (less than 18 hours) on favorable media, there is usually a predominance of long rods (8 to 12 μ) showing the marked flexibility and active creeping movements so characteristic of myxobacteria. For the most part, such cells are not markedly curved, although a few may show this property, some having a slight hook at one extremity. Occasional branched, Y-shaped individuals like those observed in cultures of *Cytophaga columnaris* by Garnjobst (1945) can be found. However, in addition to these long rods very short, sometimes almost coccoid elements, 1 to 3 μ in length, are also evident. In strains 1, 2, 3, and 6 these short elements become predominant only in cultures several days old, or on unfavorable media; in strains 4 and 5, however, they are always present in appreciable numbers at the earliest stages of development and rapidly become the predominant or even exclusive morphological form. Inspection of cultures with a predominance of short cells does not suggest that the organisms are myxobacteria: flexibility is not evident, and motility of the individual cells is hard to detect, the only indicative feature which remains being the weak refractility of the cells. The picture, both in wet mounts and in stained preparations, is strongly suggestive of a small, nonmotile, gram-negative true bacterium.

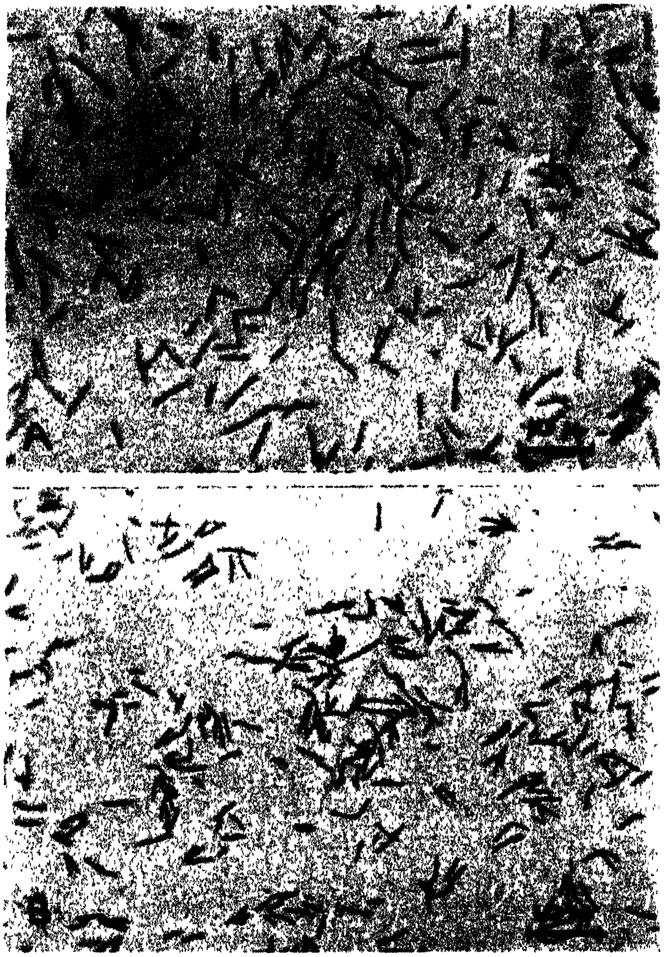

FIG. 1. VEGETATIVE CELLS OF CYTOPHAGA JOHNSONAE FROM CULTURES 14 HOURS OLD ON 0.5 PER CENT TRYPTONE AGAR

Fixed with osmic acid and stained by Winogradsky's method (1929). A. Strain 2. B. Strain 6 (var. *denitrificans*). Note the short elements in both photomicrographs.

A careful search of cultures on many different media failed to reveal the presence of microcysts.

Growth on solid media. The manner of growth on mineral agar containing finely divided chitin has already been mentioned. The rapidity and extent of swarming on this medium can be controlled to some degree by varying the agar concentration. With 1.5 to 2.0 per cent agar, the whole surface of a streaked plate becomes covered with a thin layer of cells in 36 to 48 hours; however, when the concentration of agar is reduced to 1.0 per cent, surface spreading is greatly retarded and isolated colonies are formed (figure 2A). Such colonies are largely subsurface, the cells penetrating and creeping down through the medium. After a few days the colonies become surrounded by well-defined clear areas of chitin dissolution, as shown in figure 2A. The growth is sunken below the surface of the medium, each colony lying in a shallow crater (figure 2B). This is the "etching" phenomenon, previously described as occurring on plate cultures of cellulose-decomposing cytophagas by Stanier (1942) and Fuller and Norman (1943).

When chitin-decomposing myxobacteria are streaked on nutrient agar or yeast extract agar with the customary concentrations of proteinaceous materials, a radically different type of growth is obtained. The colonies are convex, smooth and glistening with an entire edge, and at least in the earlier stages of development there is absolutely no sign of swarming. As such plate cultures age, well-isolated colonies gradually increase in diameter to as much as 5 to 10 mm, but always remain unchanged in form. On the more crowded portions of a streaked plate, however, slight swarming may appear after 5 to 6 days; the originally compact growth becomes surrounded by a small, flat, swarming periphery which spreads 2 to 3 mm from the main mass. This delayed swarming is easily overlooked, in which case there is nothing to suggest the myxobacterial nature of the culture.

Further study of growth on peptone media showed that typically myxobacterial development could be obtained, but only if the nutrient concentration was kept low. At a peptone concentration of 1.0 per cent or over, the primary development is always compact and raised with an entire edge (figure 3A). As the peptone concentration is dropped below 1.0 per cent, swarming becomes more and more evident, 0.5 per cent peptone giving rise to colonies with slightly raised center and a broad, flat, actively motile periphery (figure 3B). Finally, at peptone levels of 0.25 per cent or less, completely flat, rapidly spreading, almost invisible swarms are produced (figure 3C). Thus colony structure in these organisms is not fixed, but can be changed at will by varying nutrient concentration from a compact, eubacterial type to a diffuse, swarming, myxobacterial manner of development.

Agar concentration also plays a part in conditioning colony form on peptone media. With 1.5 to 2.0 per cent agar, there is little penetration of the medium at any level of peptone concentration; but with 1.0 per cent agar, provided that the peptone concentration is 0.5 per cent or less, growth on streaked plates tends to be largely subsurface and sunk below the level of the surrounding medium. With higher peptone concentrations, a reduction in the agar concentration produces no changes.

There appear to be two factors which operate to cause these variations in the manner of growth. In the first place, it is evident that the characteristic creeping motility of these organisms can be inhibited by high concentrations of nutrients. This phenomenon can be corroborated by microscopic exami-

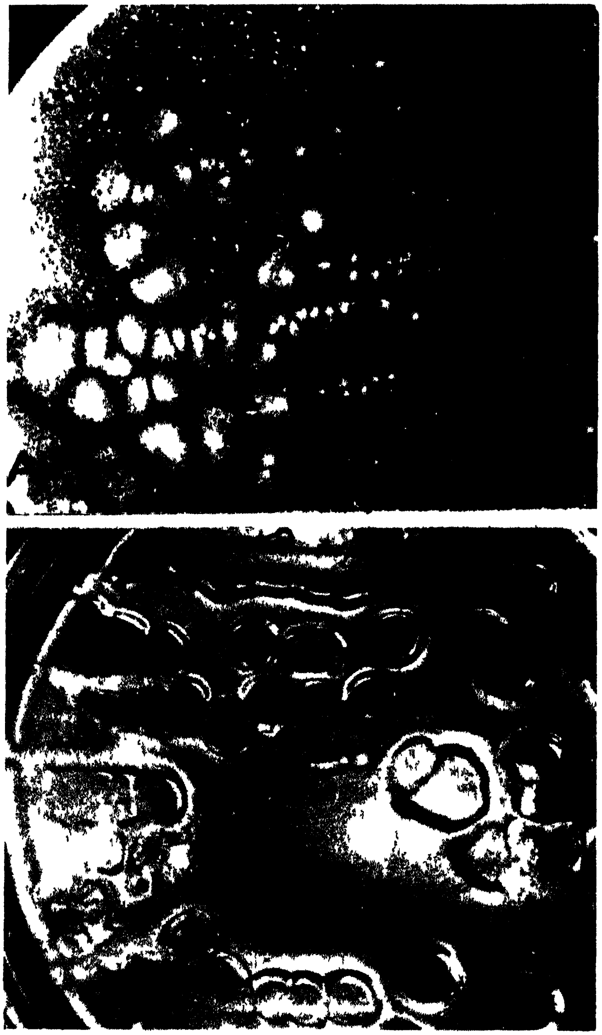

FIG. 2. CYTOPHAGA JOHNSONAE, STRAIN 1

Streaked plates on mineral 1.0 per cent agar containing a suspension of chitin, incubated for 6 days at 25 C A Photographed by transmitted light to show colony structure and dissolution of chitin. B. Photographed by reflected light to show the shallow craters formed by the colonies.

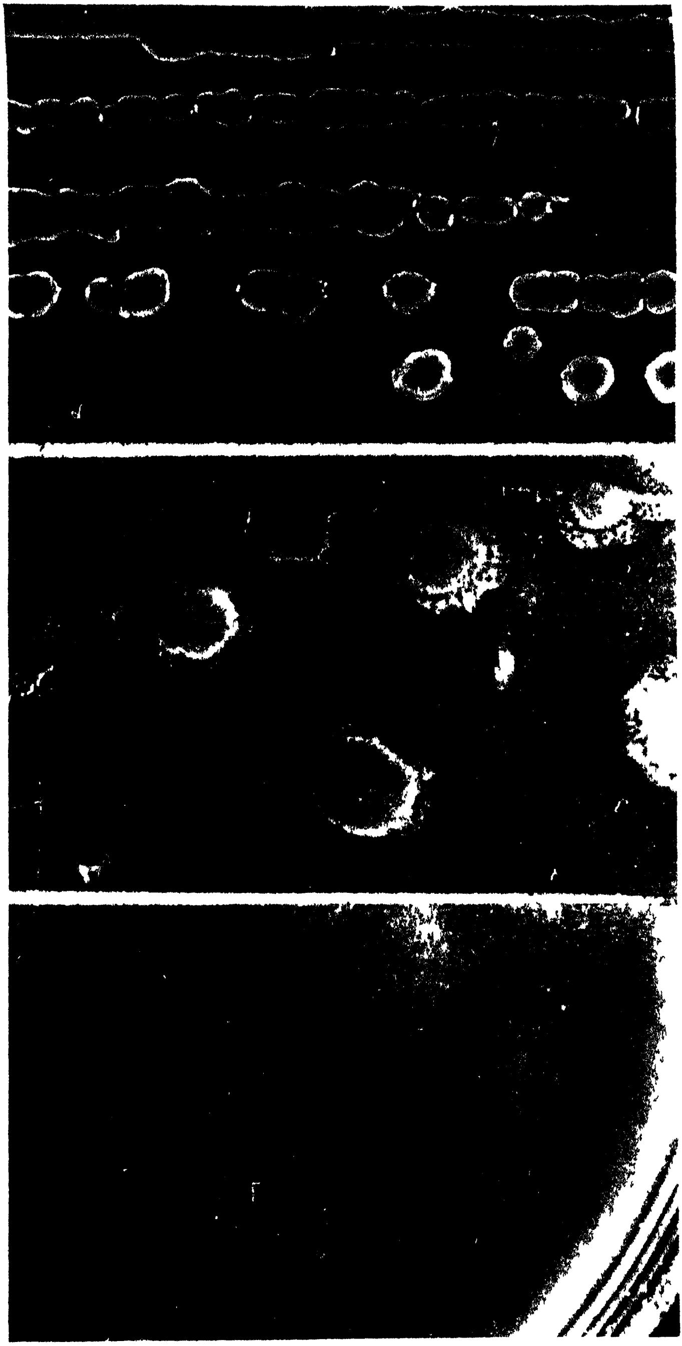

FIG. 3. CYTOPHAGA JOHNSONAE, STRAIN 1

Streaked plates on tryptone agar, incubated for 6 days at 25 C, showing the effect of nutrient concentration on colony form. A Plate with 2 0 per cent tryptone. B. Plate with 0.5 per cent tryptone. C. Plate with 0 25 per cent tryptone.

nation: cells from nonswarming colonies show no signs of individual flexing or creeping movement. As a result of the inhibition of movement, colony growth becomes a passive phenomenon, as it is in most true bacteria, and a similar colony structure is produced. The second factor influencing colony form is the ability of the cells, provided that their movement is not inhibited by nutrient concentration, to penetrate a weak agar gel. Cells deposited on the surface of a 1.0 per cent agar gel can move freely through the medium as well as across it, and thus colonies approach in form a hemisphere buried in the medium with its plane surface at the surface of the agar. On the other hand, when the agar is sufficiently firm to offer a barrier to the penetration of the cells, organisms deposited on its surface can only move out in a flat swarm, and the resulting colony approximates in form a plane of cells. The three possibilities are shown diagrammatically in figure 4.

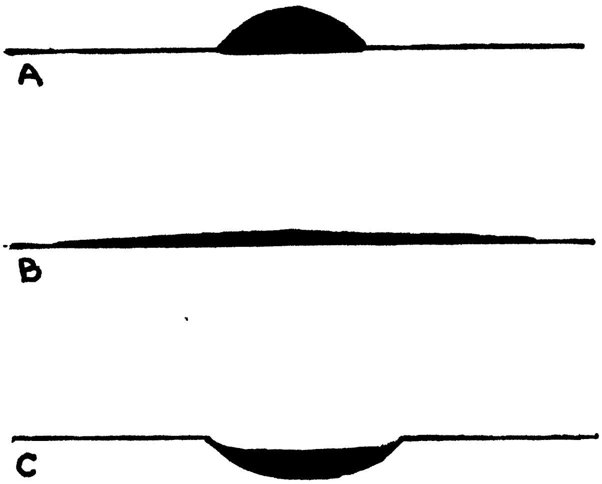

FIG. 4. DIAGRAMMATIC CROSS SECTIONS OF THE THREE TYPES OF COLONIES PRODUCED BY C. JOHNSONAE

A. Compact type, resulting from inhibition of movement by high nutrient concentration. B. Diffuse, rapidly swarming colony on medium with low nutrient concentration and a firm gel. C. Sunken type, embedded in the substrate, on medium with a low nutrient concentration and a weak gel.

Nutrient requirements and physiology. All strains can develop in a mineral medium containing the necessary salts together with ammonia nitrogen and a suitable carbon compound. The carbohydrates are the energy sources of preference. Heat sterilization does not render reducing sugars toxic. Development in such simple media is rather slow and never very abundant; it can be greatly accelerated by the addition of small amounts of peptone or yeast extract. It is possible, however, to maintain all strains indefinitely in a mineral-carbohydrate medium without such additions. A wide range of simple carbohydrates is attacked; of the polysaccharides, chitin and starch are utilized by all, cellulose and agar by none. Some strains, notably 4, develop much better on disaccharides than on their constituent monosaccharides. In addition to ammonia, nitrate but not nitrite can be used as a nitrogen source.

Maximal development occurs on complex nitrogenous media, Difco tryptone being particularly favorable. Milk and peptone gelatin are rather poor substrates; this is apparently due to the high concentration of nutrients, since vigorous development can be obtained with diluted milk. In general, concentrations of peptone and other proteinaceous materials of 1.0 per cent or over are deleterious, and even if, as sometimes proves the case, development is abundant, viability turns out to be poor. Thus good growth will occur on nutrient agar or 1.0 per cent tryptone agar, but cultures on these media are often dead after 2 to 3 weeks, whereas cultures on 0.25 to 0.5 per cent tryptone agar will remain viable for 2 months or longer.

All strains are actively proteolytic, although this property is not evident on the customary media because of poor growth. On plates of plain agar containing 10 per cent milk, the growth is surrounded by wide zones of casein dissolution in 2 to 3 days. Bacteriolytic enzymes of the type found in many higher myxobacteria are absent; all strains proved without effect when tested against a strain of *Aerobacter aerogenes* readily lysed by members of the *Myxococcaceae*.

The metabolism is strictly oxidative. There are no signs of carbohydrate fermentation, and in the absence of nitrates development only occurs aerobically. In the presence of nitrates, two strains can grow anaerobically, using nitrate as a hydrogen acceptor in the place of free oxygen. One of these (strain 4) reduces nitrate only to nitrite, and the resulting anaerobic growth is somewhat sparse. The other (strain 6) is an active denitrifier, which grows abundantly with nitrates under anaerobic conditions and gives rise to considerable gas production.

TAXONOMIC POSITION

It is clear that in spite of the morphological and cultural anomalies mentioned above, the strains studied are myxobacteria which belong, on the absence of fruiting bodies and microcysts, in the family *Cytophagaceae*. For reasons which will be made clear in the subsequent discussion I prefer to place them in the genus *Cytophaga*, although on the basis of their morphology and nutrient requirements, respectively, they might be assigned to either of the two new genera of nonfruiting myxobacteria, *Promyxobacterium* (Imsenecki and Solntzeva, 1945) or *Flexibacter* (Soriano, 1945).

Although there are minor differences between strains in cell size, carbon requirements, and action on nitrates, none of the distinctions seem sufficiently important or well correlated to justify creation of more than one species. A possible exception is strain 6, with its pronounced denitrifying abilities. However, this strain is very similar in other respects to the remaining strains, and hence it seems wiser to regard it simply as a variety. Since all strains appear to correspond to the chitin decomposers described by Johnson (1932) as nonfruiting, amicrocystogenous myxococci, they may be appropriately grouped under the designation *Cytophaga johnsonae* n. sp., with strain 6 as a special variety, var. *denitrificans*. The descriptions follow:

Cytophaga johnsonae, n. sp.

Morphology: Thin, weakly refractile rods of even width and very variable length, with rounded ends. Dimensions 0.2 to 0.4 by 1.5 to 15 μ. Long rods (8 to 10 μ) predominate in very young cultures on dilute media, but in most strains soon give place to shorter, sometimes almost coccoid, elements as cultures age. Branched cells occasionally occur in young cultures. Creeping motility on media of suitable nutrient concentrations. Gram-negative. Cells do not contain volutin or fat bodies, and stain evenly with Giemsa and Winogradsky's stains.

Peptone agar plate: Growth smooth, glistening, translucent, butyrous, and bright yellow in color. The appearance of growth varies greatly with peptone concentration. With 0.5 per cent or less, a flat, thin, spreading, inconspicuous, pale yellow swarm of the characteristic myxobacterial type is formed. With higher concentrations, swarming decreases or completely disappears, and the colonies are raised, convex, and confined, with an entire edge. With agar concentrations of 1.0 per cent or less and a low nutrient concentration there is marked penetration of the medium with decreased lateral swarming.

Mineral chitin agar plate: Smooth, glistening, butyrous, translucent, pale yellow growth, accompanied by dissolution of the suspended chitin. Swarm extension is extremely rapid on 2 per cent agar, but on 1 per cent agar discrete colonies are formed owing to penetration of the medium and decrease of surface swarming. Development is not as profuse as on peptone agar.

Plain agar with 10 per cent milk: abundant, spreading growth with dissolution of the casein.

Peptone gelatin stab: Scanty growth with some strains followed by very slow liquefaction. Others fail to develop.

Liquid media (peptone water, yeast extract, mineral media with an oxidizable carbohydrate): Turbidity with pronounced silkiness. After several days a slimy coherent sediment is formed, and in the course of time the suspended cells tend to sink to the bottom, leaving the upper layers of the medium clear. Some strains produce a pellicle.

Milk: Very slow peptonization. One strain fails to develop.

Peptone water with 3 per cent NaCl: No growth.

Utilizable energy sources: Arabinose, xylose, glucose, galactose, mannose, lactose, maltose, sucrose, cellobiose, raffinose, starch, inulin, chitin, asparagine (all strains). Succinate is used by three strains out of five, fumarate and malate by one. Cellulose, mannitol, dulcitol, lactate, acetate, malonate, tartrate, citrate, and alanine are not attacked. Proteinaceous materials can also serve as the energy source in the absence of carbohydrates.

Utilizable nitrogen sources: Nitrate, ammonia, alanine, and peptones. Not nitrite or glycine.

Casein hydrolyzed by all strains, gelatin by most. Bacteriolytic enzymes not produced.

Catalase produced.

Indole not formed.

Strictly aerobic, except for one strain which can develop anaerobically to a slight extent in the presence of nitrate (reduced to nitrite).

Optimum temperature: 25 to 30 C.

Source: Soil and mud.

Habitat: Probably common in soil.

Cytophaga johnsonae var. denitrificans

Morphology: Similar to *C. johnsonae.*

Peptone agar plate: Similar to *C. johnsonae.*

Mineral chitin agar plate: Similar to *C. johnsonae,* but attack on chitin very weak.

Plain agar with 10 per cent milk: Similar to *C. johnsonae.*

Peptone gelatin stab: Very slight growth, followed by slow liquefaction.

Liquid media: Similar to *C. johnsonae.*

Milk: Slow peptonization.

Peptone water with 3 per cent NaCl: No growth.

Utilizable energy sources: Arabinose, xylose, glucose, galactose, mannose, maltose, sucrose (growth weak and delayed), starch, inulin (weak), chitin (weak), succinate, fumarate, and asparagine. Lactose, cellobiose, raffinose, cellulose, mannitol, dulcitol, sorbitol, lactate, acetate, malate, malonate, tartrate, citrate, and alanine not attacked.

Utilizable nitrogen sources: Same as *C. johnsonae.*

Gelatin and casein hydrolyzed. Bacteriolytic enzymes not produced.

Catalase produced.

Indole not formed.

Strictly aerobic except in the presence of nitrate. Can develop abundantly under anaerobic conditions by employing denitrification as a mechanism for oxidative metabolism. Such growth is accompanied by vigorous gas production and a transient appearance of nitrite.

Optimum temperature: 25 to 30 C.

Source: Compost.

Habitat: Probably widely distributed in soil.

DIFFERENTIATION OF NONFRUITING MYXOBACTERIA FROM ROD-SHAPED TRUE BACTERIA

When the taxonomic recognition of nonfruiting myxobacteria was first proposed (Stanier, 1940), the differentiation of these organisms from rod-shaped true bacteria appeared fairly simple. This is still true of the microcystogenous forms (genus *Sporocytophaga*); microcyst formation is a highly distinctive developmental process, never found in eubacteria. On the other hand, it now appears from the work of Imsenecki and Solntzeva (1945) as well as from the present study that myxobacteria which exist only in the vegetative state (family *Cytophagaceae*) may well simulate true bacteria under certain circumstances.

The two species described by Imsenecki and Solntzeva (1945) under the names *Promyxobacterium flavum* and *P. lanceolatum* were for some time regarded by those authors as nonsporeforming true bacteria, on account of the shortness of the cells and the, for myxobacteria, uncharacteristic manner of growth. *Cytophaga johnsonae*, if examined solely from cultures on the customary concentrated media, would appear to be a short, gram-negative, nonmotile rod classifiable according to the system of Bergey *et al.* in the regrettable genus *Flavobacterium;* indeed, were it not for the fact that it originally developed on a special chitin medium which revealed at once the swarming growth, this mistake might easily have been made. In view of such findings, it seems possible that the ill-defined eubacterial genera which contain gram-negative, nonsporeforming, supposedly nonmotile (i.e., nonflagellated) rods—*Bacterium*, *Achromobacter*, and *Flavobacterium* of Bergey *et al.*—may be harboring unsuspected myxobacteria of the *Cytophaga* type which were studied under conditions inapt to reveal their true properties. Since the differentiation of myxobacteria from true bacteria is a matter of some practical as well as theoretical importance, it seems worth while to discuss, in the light of recent work, what is now known about the general properties of imperfect myxobacteria and to indicate features likely to be helpful in the recognition of these organisms.

Morphology. The earlier concept that the cells of the cytophaga group are slender, spindle-shaped rods with pointed ends must now be abandoned. Derived from the study of the classical cellulose-decomposing species in stained preparations, this concept is not properly speaking true even of these organisms, since, as I have previously remarked (1942), when examined *in the living state* they are rods of even width with slightly rounded ends. The same description applies to all other species so far studied. It is now also evident that the length of the cells may be extremely variable, not all species having the long, slender cells characteristic of the cellulose- and agar-decomposing groups. The two species described by Imseneeki and Solntzeva (1945) are almost coccoid under some conditions; in *Cytophaga columnaris*,[3] of which Garnjobst (1945) has given an admirable morphological description, very short cells occur intermingled with longer elements; in *Cytophaga johnsonae* the cells may be long and slender or short and almost coccoid depending on the conditions of cultivation and the age of the culture. The possibility exists that in all cytophagas the basic cellular units are short, and that the length of the vegetative entities is an expression of the degree of chain formation. In any case, there is no clear distinction between these organisms and rod-shaped eubacteria on the basis of cell shape *alone.* Two properties of the cell are, however, very strong indications of membership in the cytophaga group. One is marked flexibility, as shown by the presence of irregularly curved, horseshoe-shaped, or ringed vegetative elements. This character will naturally not be evident if the cells are very short, and even in long-celled cultures it is not invariably conspicuous. The other feature is the relatively weak refractility of living cytophaga cells, due to the absence of a cell wall. Imsenecki and Solntzeva (1945) suggest that this is less marked in short-celled types, but it has proved a constant feature in all the species that I have examined. As far as staining reactions are concerned, it may be noted that all members of the cytophaga group are gram-negative. Some species have a tendency to stain rather poorly with basic aniline dyes, but this is not universally true. With the exception of the microcystogenous species during the process of microcyst formation (Krzemieniewska, 1930), the cytophagas do not show discrete chromatinic structures when stained by ordinary methods. This has been interpreted by Imsenecki and Solntzeva (1945) as indicating that the chromatinic material is diffuse, a feature which in their opinion serves as a link with the true bacteria, in supposed contrast to the higher myxobacteria which have discrete chromatinic structures. It may be remarked that in all eubacteria so far properly examined discrete chromatinic structures have been found, although they are rarely demonstrable by direct staining with basic aniline dyes because of the strongly basophilic nature of the cytoplasm (cf. Robinow, 1945, for a discussion). Since the cytology of the cytophaga group has not yet been investigated in detail from the standpoint of nuclear structure, it seems decidedly premature, in view of the above-mentioned findings with true bacteria, to describe the distribution of chromatin in them as diffuse.

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[3] The systematic position of this interesting species, an important pathogen of fresh-water fishes, is not entirely clear. Ordal and Rucker (1944) have claimed discovery of fruiting body formation in strains which appear to be similar to those studied by Garnjobst.

Motility. The most decisive distinguishing property of nonfruiting myxobacteria vis-à-vis true bacteria is their nonflagellar creeping movement. So far, permanently immotile members of the *Cytophagaceae* have not been described, nor is it likely that they would be recognizable as such. On the other hand, the present work demonstrates that creeping motility is not invariably evidenced by organisms potentially capable of it. It thus becomes necessary to define as far as possible the conditions favorable for bringing this important characteristic to light.

Of primary importance is the use of dilute media for cultivation of the organisms, a point mentioned by Soriano (1945). Motility can usually be discerned in wet mounts by examining cells adherent to the slide or cover slip. Since all known myxobacteria are strict aerobes, a fairly light suspension should be used for such examinations; otherwise movement will soon cease owing to depletion of the oxygen supply. Particularly if the vegatative units are small, a convincing demonstration of creeping movement in wet mounts may prove difficult. In such cases, a variant of the usual hanging block technique, first used to study swarming by the marine agar-decomposing cytophagas (Stanier, 1940), may be applied. A loopful of melted agar is spread on a sterile cover slip and inoculated, after solidification, at one or two spots with a mass of young cells from the tip of a needle. A dilute nutrient agar may be used, although for the chitin-decomposing strains equally satisfactory results can be obtained with nonnutrient agar. The inoculated cover slip is mounted over a moist chamber, incubated at a suitable temperature and examined at intervals. After a variable period, spearheads of cells begin to creep out from the smooth periphery of the artificial microcolony, and usually within 2 to 3 hours the whole edge breaks down into a

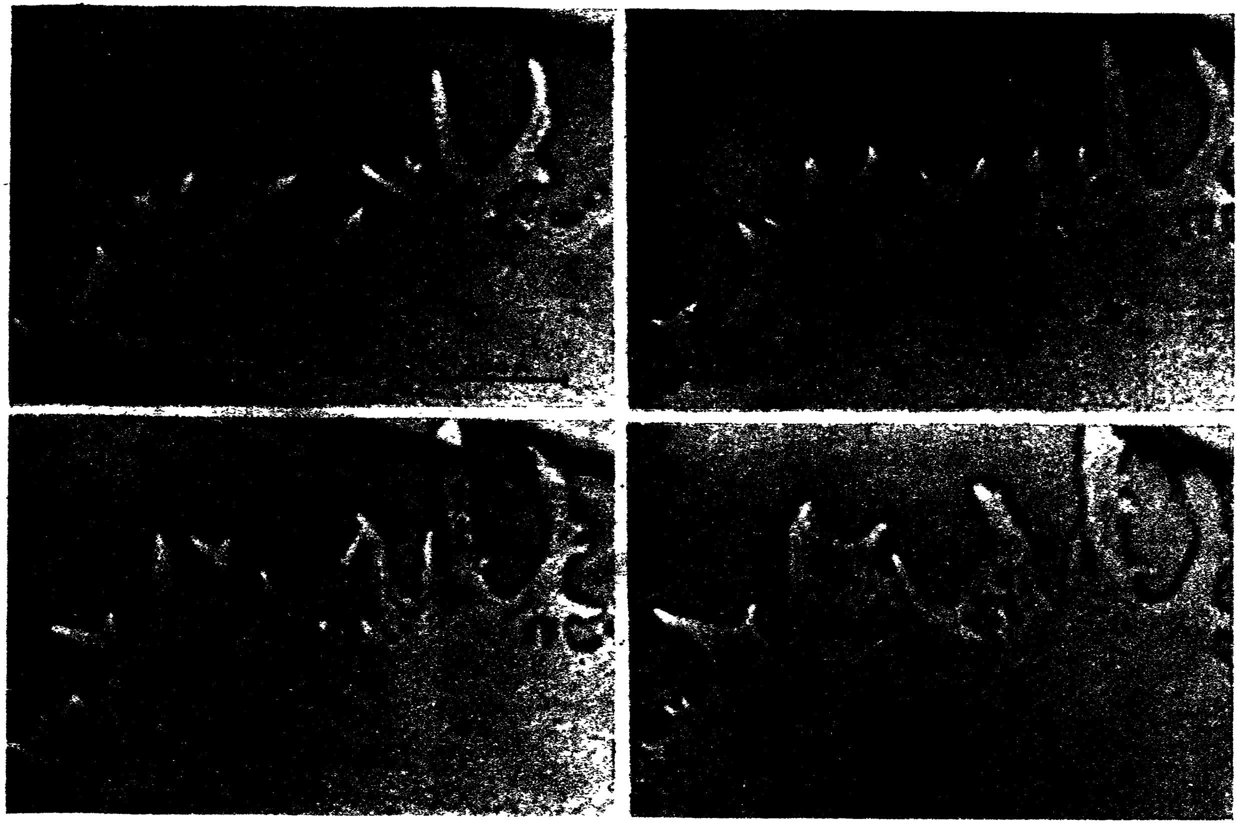

FIG. 5. C. JOHNSONAE, STRAIN 2

Swarming edge of an artificial microcolony on 2 per cent agar without nutrients, photographed at intervals. A. 11:50 A.M. B. 11:57 A.M. C. 12:05 P.M. D. 12:15. P.M.

series of actively moving columns, which spread out and interlace with one another over the surrounding agar. Provided that wet mounts have shown the absence of flagellar locomotion, this constitutes a decisive proof of creeping motility. A series of consecutive photomicrographs of swarming from such an artificial microcolony of *C. johnsonae* are shown in figure 5.

Cultural characters. Certain cultural characters offer hints to the observant for the identification of imperfect myxobacteria. The most suggestive features are flat, rapidly spreading growth on solid media, the penetration of weak agar gels by the cells, and the etching of the surface of agar media. These properties are not, however, invariably shown; they were apparently absent in the cultures studied by Imsenecki and Solntzeva (1945), and are not found in *C. hutchinsoni* (Stanier, 1942) or in *C. johnsonae* when grown on concentrated media.

Other properties. There are no really distinctive nutritional, biochemical, or physiological characters common to all nonfruiting myxobacteria. Strict aerobiosis and a rather low temperature optimum (30 C or less) are common; but an adequate search for myxobacteria with other oxygen and temperature relationships has so far not been made, and these particular properties are widely found in the eubacterial groups most likely to be confused with the nonfruiting myxobacteria. Although there is a definite group tendency toward the decomposition of complex polysaccharides, this is by no means universal; *C. columnaris* (Garnjobst, 1945) and the *Promyxobacterium* spp. described by Imsenecki and Solntzeva (1945) lack this property. In addition, I have examined several strains of imperfect myxobacteria isolated by chance from soil which failed to attack any of the polysaccharides tested (agar, chitin, cellulose) except starch. The inability to use any source of carbon save carbohydrates is a highly distinctive feature of the classical cellulose-decomposing cytophagas, but is not found in the more recently described species. The members of the group are markedly heterogeneous in their relation to growth factors: some (viz., the soil cellulose-decomposing group, *C. johnsonae* and the *Promyxobacterium* spp. of Imsenecki and Solntzeva) are capable of satisfactory growth with ammonium salts and a suitable carbon source, but others definitely require growth factors, although in no case have the exact needs been worked out.

RECENT PROPOSALS ON THE TAXONOMY OF THE NONFRUITING MYXOBACTERIA

When the taxonomic recognition of the nonfruiting myxobacteria was first proposed (Stanier, 1940, 1942), the suggestion was made that the microcystogenous species should be included in the family *Myxococcaceae* as a new genus, *Sporocytophaga*, and that a new family, the *Cytophagaceae*, with a single genus *Cytophaga* Winogradsky emend. should be adopted for the amicrocystogenous species. As originally defined by Winogradsky (1929), the genus *Cytophaga* was characterized in part by obligate cellulose decomposition, a physiological property which proved no longer valid following the discovery that the species included could attack other carbohydrates. The family *Cytophagaceae* and the genus *Cytophaga* were then redefined (Stanier, 1942), on purely morphological grounds, as containing organisms with the characteristic features of myxobacterial vegetative cell structure, but incapable of forming either fruiting bodies or microcysts.

Recently two modifications in the taxonomy of this group have been proposed. Imsenecki and Solntzeva (1945) suggest a family *Promyxobacteriaceae*, defined as containing myxobacteria which fail to form fruiting bodies, and divided into three genera: *Promyxobacterium*, *Cytophaga*, and *Sporocytophaga*. The new genus *Promyxobacterium*, created for two species described by Imsenecki and Solntzeva, is stated to consist of organisms whose vegetative cells are rod-shaped with rounded ends, in supposed contradistinction to the other two genera in which the vegetative cells are stated to be long, thin, and slightly bent with pointed ends. The proposed family *Promyxobacteriaceae*, which would unite all nonfruiting myxobacteria and separate them from myxobacteria at a higher level of biological organization as expressed by the formation of discrete fruiting bodies, has much to recommend it. The possible merits of this arrangement have been assessed previously, and it has been pointed out that the decision between such a taxonomic scheme and one which places the genus *Sporocytophaga* in the *Myxococcaceae* must remain more or less a matter of arbitrary choice (Stanier, 1942).

On the other hand, the genus *Promyxobacterium* as it is defined by Imsenecki and Solntzeva would, I feel, prove completely unworkable, since the supposed morphological distinction between organisms of this type and *Cytophaga* species is not a clear-cut one. When examined in the living state, cells of the classical cellulose-decomposing cytophagas are of even width, with ends which are only slightly pointed, if at all. In the last analysis, the difference between *Promyxobacterium* and *Cytophaga* appears to be one of cell length, which is not really tenable. In *Cytophaga johnsonae* and *C. columnaris*, the cells may be short and *Promyxobacterium*-like or long and *Cytophaga*-like, and, depending on cultural conditions, one or the other type can predominate.

The taxonomic proposals of Soriano (1945) are more far-reaching, and comprise part of a scheme for rearranging the major groups of bacteria. Only the pertinent sections of his suggested changes will be discussed here. He proposes that the order *Myxobacteriales* should be kept as originally conceived for fruiting myxobacteria alone, and that a new order *Flexibacteriales* should be created to contain all bacteria with flexible, rod-shaped cells and creeping motility that are incapable of forming fruiting bodies. In this order he assembles three families: the *Cytophagaceae*, containing the genera *Cytophaga* and *Sporocytophaga*; the *Beggiatoaceae*, containing the genus *Beggiatoa*; and the *Flexibacteriaceae*, containing the genus *Flexibacter*. The latter family and genus were created by Soriano for several nonfruiting, amicrocystogenous myxobacteria discovered by him that develop on peptone and meat extract media and are unable to attack cellulose. The three families are defined and distinguished primarily on nutritional grounds as follows:

Flexibacteriaceae. Forms which do not contain granules of sulfur and are incapable of attacking cellulose.

Cytophagaceae. Flexible bacteria, spindle-shaped at some period of their development, which attack cellulose and may or may not produce cysts.

Beggiatoaceae. Flexible, filamentous forms, without photosynthetic pigments, containing sulfur granules within the cells.

Two principal criticisms can be made of the proposals of Soriano. The first, and less serious, is that so many lines of evidence link the nonfruiting myxobacteria with the higher myxobacteria—all the common features of vegetative cell structure, together with the mode of mycrocyst formation in the *Sporocytophaga* species—that an ordinal separation is too drastic. The second is that the order *Flexibacteriales* is not in itself a natural assemblage, since the families *Flexibacteriaceae* and *Cytophagaceae* differ radically in structure and organization from the *Beggiatoaceae*. As is now commonly recognized, the latter family comprises colorless counterparts of blue-green algae belonging to the family *Oscillatoriaceae*. Since the practice in other algal groups of placing colorless genera and families near morphologically related photosynthetic forms is well established (cf. Lwoff, 1943, and Pringsheim, 1941, for discussions of colorless flagellates), there is no justification except a false bacteriological tradition for not following the same procedure in the case of the *Beggiatoaceae* and removing them from the bacteria altogether. If this is done, Soriano's order *Flexibacteriales* becomes a repository for nonfruiting myxobacteria, equivalent to Imsenecki and Solntzeva's *Promyxobacteriaceae*, or to my family *Cytophagaceae* with the addition of the genus *Sporocytophaga*.

The problem of generic and familial segregations among the nonfruiting myxobacteria is a difficult one. If these organisms really comprise as large and varied a group as appears to be the case from recent work, a single genus and family may well prove insufficient to include them all; but before additional genera and families are accepted, careful thought should be given to the selection of significant, mutually exclusive differential characters. As already mentioned, the *Promyxobacterium-Cytophaga* division along morphological lines proposed by Imsenecki and Solntzeva (1945) is untenable. We are faced here with a taxonomic difficulty which has previously been encountered in the true bacteria; namely, that of finding foolproof physiological differential characters which can be used to split up a group of strict aerobes sharing common morphological properties. Viewed in this light, Soriano's proposed *Flexibacter-Cytophaga* division on the basis of ability or inability to attack cellulose is simply a continuation of the policy expressed in the generic pairs *Vibrio-Cellvibrio* and *Pseudomonas-Cellulomonas*. In all these cases, the mutational loss of a single hydrolytic enzyme, an event not too difficult to envisage in the light of recent work on the biochemical genetics of microorganisms, would be sufficient to change the generic position of an organism. An additional obstacle to such generic separations, clearly evident in the *Vibrio-Cellvibrio* group, is provided by the intergrading species endowed with a general ability to attack complex polysaccharides, but lacking the specific enzyme cellulase (e.g., the agar-decomposing vibrios).

Until nonfruiting myxobacteria have been more extensively studied, the genus *Cytophaga* appears to me to provide an adequate taxonomic pigeonhole for all known amicrocystogenous species. Mention may be made here, however, of one significant physiological property which might be applied to give a generic segregation roughly equivalent to that suggested by Soriano; namely, the type of organic compound which can be used as a source of energy. The classical cellulose-decomposing cytophagas are highly restricted in this respect, being unable to develop in the absence of carbohydrates. All other known species of nonfruiting myxobacteria can use peptides, and in certain cases other simpler organic compounds, to fulfill their energy requirements. A division into two genera, *Cytophaga* and *Flexibacter*, on this basis would, I believe, prove more satisfactory than one which rested solely on the ability or inability to hydrolyze cellulose.

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SUMMARY

Amicrocystogenous nonfruiting myxobacteria commonly occur as a component of the chitin-decomposing microflora in aerobic chitin enrichment cultures.

Several strains of these organisms have been isolated and studied. Although atypical in some respects from the morphological and cultural standpoints, they are all representatives of the genus *Cytophaga*. The nutrition is unspecialized, and good growth occurs on a wide variety of media. Only slight differences between strains were found, and they are consequently regarded as representatives of a single species, *Cytophaga johnsonae*, n. sp. One strain characterized by pronounced denitrifying abilities is separated as a variety, var. *denitrificans*.

The taxonomy of the nonfruiting myxobacteria and the problem of their differentiation from rod-shaped eubacteria are discussed in the light of recent work.

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COMPARATIVE DISTRIBUTION AND POSSIBLE PATHOGENICITY OF PARACOLOBACTRUM SPECIES IN AN AREA HIGHLY ENDEMIC FOR ENTERIC INFECTIONS[1]

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In an area where enteric infections are highly endemic, the etiological agents of the infections are not always clearly evident. For example, the percentage of specimens positive for commonly accepted intestinal pathogens in over 800 stool specimens examined in North Africa from May through September, 1943, fell from 70 per cent during May and June, when gastroenteritis among American troops was at its maximum, to 30 per cent and then to 10 per cent in the months following.[3] Inasmuch as the majority of specimens examined were from cases of enteritis, it was felt that the commonly accepted pathogens were not always the cause of these disorders, and it was decided to investigate the comparative distribution of *Paracolobactrum* species. Such a study, it was believed, would yield additional information concerning possible pathogenicity of that group of organisms and would, in addition, furnish data on their relative frequency in normal individuals in an endemic area. The investigation was therefore initiated in January, 1944, and specimens were collected until May of that year in Casablanca, North Africa, the cultural and serological work being continued until August, 1944, following transfer of the organization to Rome, Italy. In this paper, the nomenclature for the paracolon organisms proposed by Borman, Stuart, and Wheeler (1944) will be used.

METHODS OF INVESTIGATION

The native Arab population of this area, living as it does under primitive sanitary conditions, is obviously the reservoir of intestinal pathogens. It was desirable, therefore, in order to determine what organisms were normally present, to obtain feces specimens from this group of people. This was done through the co-operation of a local Arab hospital, which furnished specimens from newly admitted patients with disorders other than intestinal ones. As a further control on normally occurring organisms, specimens from healthy American soldiers or from Italian prisoner of war food handlers, living under Army sanitary conditions, were obtained. Specimens from these sources were collected during the same period that specimens from hospital patients with enteritis were examined.

All specimens were planted on eosin methylene blue agar, SS agar, and selenite-f broth immediately on arrival. After 24 hours' incubation, suspicious colonies on the plates were transferred to Kligler agar slants. Material from the selenite-f broth was planted on SS agar, and colorless colonies were picked the following day. All cultures so obtained were tested biochemically. Commonly accepted pathogens were identified serologically, and preliminary studies on the antigenic relationships of the most frequently occurring *Paracolobactrum* species were made.

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[1] This study was made in the laboratory service of the 6th General Hospital, U. S. Army, Casablanca, North Africa, and Rome, Italy.

[2] Captain, U. S. A. Reserve, Retired. With the technical assistance of T/5 Wayne Easterday.

[3] Data collected by the author while Chief of the Bacteriology Section, 1st Medical Laboratory, U. S. Army, Bizerte, North Africa.

DISTRIBUTION OF ORGANISMS

Table 1 shows the distribution of the most frequently isolated slow- and non-lactose-fermenting organisms obtained. As was expected, the greatest number of positive isolations was made from hospital cases, amounting to 81.1 per cent of the hospital specimens submitted, as compared to 61.1 per cent positive normal Arab specimens, and 32.6 per cent positive normal American specimens.

Salmonella species were isolated from 13.5 per cent of the hospital specimens, from 5.5 per cent of normal Arab specimens, and from 8.7 per cent of normal American specimens. *Shigella* species were isolated from only 8.1 per cent of hospital specimens, from 18.5 per cent of normal Arab specimens, and from 4.3 per cent of normal American specimens. Though these figures indicate an apparent lack of significance in the distribution of the commonly accepted pathogens, there can be little question that *Salmonella* and *Shigella* species isolated from hospital patients were in fact the causes of the enteric infections. Most of the positive hospital stools were pathological, containing blood or mucus or both, though some were of a more normal appearance. *Paracolobactrum* species were isolated in only a few cases in association with the ordinary pathogens. The high percentage of *Salmonella* and *Shigella* species isolated from normal American and normal Arab specimens, whose stools in most cases were normal in appearance, indicates a high frequency of the carrier state, a condition which could be expected in a highly endemic area.

There was, on the other hand, a marked difference in the distribution of *Paracolobactrum* species isolated from the three sources. Organisms of that group were obtained from 62.2 per cent of hospital specimens, from 42.6 per cent of normal Arab specimens, and from 19.5 per cent of normal American specimens.

In addition to the organisms recorded in table 1, anaerogenic organisms possibly belonging to the genus *Proshigella* were isolated but were not of significance either in numbers or distribution. Numerous *Proteus* species were also isolated, being found in 29.6 per cent of normal Arab specimens, in 10.8 per cent of hospital specimens, and in 2.2 per cent of normal American specimens.

Table 2 shows the distribution of the *Paracolobactrum* cultures obtained. *P. aerogenoides* cultures were found about five times as often in hospital patients as in normal Arabs, and about seven times as often as in normal Americans. *P. intermedium* cultures were isolated about twice as often from hospital patients as from normal Arabs, and about three times as often as from normal Americans. *P. coliforme*, however, was isolated only twice as often from hospital patients as from normal Americans, and only half as often as from normal Arabs.

CULTURAL AND ANTIGENIC CHARACTERISTICS

Cultural characteristics and antigenic relationships of the *Paracolobactrum* cultures isolated are shown in table 3. Most of the cultures were held on nonfermentable carbohydrate media, citrate agar (Simmons), and urea agar (Christensen, 1946) for 3 weeks in the preliminary studies. Hydrogen sulfide production was determined in Kligler's medium. Indole production was determined by means of Kovac's reagent, and acetylmethyl-carbinol production, because of the lack of more sensitive reagents, was determined by use of 10 per cent potassium hydroxide solution. Semisolid agar at room temperature and at 37 C was used to determine motility. Following the preliminary study, made immediately upon isolation of the cultures, all reactions were repeated and additional tests were added. Most cultures were held on nonfermentable carbohydrate broths in the second series for 40 days.

TABLE 1

Comparative distribution of Paracolobactrum, Salmonella, and Shigella species

SOURCE OF SPECIMEN	NUMBER OF SPECIMENS	POSITIVE SPECIMENS		SPECIMENS CONTAINING					
				Paracolobactrum		*Salmonella*		*Shigella*	
		No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Hospital patients	37	30	81.1	23	62.2	5	13.5	3	8.1
Normal Arabs	54	32	61.1	23	42.6	3	5.5	10	18.5
Normal Americans	46	15	32.6	9	19.5	4	8.7	2	4.3

TABLE 2

Distribution of Paracolobactrum cultures

SOURCE OF CULTURE	P. AEROGENOIDES		P. INTERMEDIUM		P. COLIFORME	
	No.	Per cent	No.	Per cent	No.	Per cent
Hospital patients	11	29.7	7	18.9	6	16.2
Normal Arabs	3	5.6	5	9.3	16	29.6
Normal Americans	2	4.1	3	6.5	4	8.7

Percentages are in terms of number of specimens examined. See table 1.

For antigenic studies, one strain from each of the largest cultural groups was used for antiserum production. This study was to have included complete antigenic comparison among themselves of the *Paracolobactrum* organisms isolated, as well as comparison with known *Salmonella* and *Shigella* antigens. The investigation was terminated, however, before complete antigenic and cultural studies were completed. It will be noted that cultural characteristics of only 20 *P. coliforme* organisms are given in table 3, though a total of 26 cultures were isolated, as shown by their IMViC reactions. Lack of time prevented more complete study of the 6 cultures not listed, and also of cultures listed which were not tested on all media given in the table.

TABLE 3

Cultural characteristics and antigenic relationships of Paracolobactrum cultures

ORGANISMS	GROUP	NO. OF STRAINS	H₂S	INDOLE	V. P.	CITRATE	UREA	GELATIN	MOTILITY	GLUCOSE MANNITOL MALTOSE	LACTOSE	SUCROSE	SALICIN	NO OF CULTURES ANTIGENICALLY RELATED
P. aerogenoides	1	16	−	−	+	+	+	9+ 6–8 days 7–0	15+ 1−	+	+ 2–8 days	+	7+ 2–3 days 1− 8–0	13
P. intermedium	1	12	+	−	−	+ 1–4 days	+	8− 4–0	11+ 1−	+	+ 4–7 days	+	− or + 11–20 days	4 (5–0)
	2	3	−	−	−	−	1+ 2−		1+ 2−	+	+ 5–14 days	− or + 4 days		
P. coliforme	1	9	−	+	−	−	−	−	9+ 1−	+	−	−	− or + 2–4 days	2
	2	3	−	+	−	−	−	−	1+ 2−	+	± 9–35 days	−	− or + 6 days	
	3	8	−	+	−	−	−		±	+	− or ± 2–4 days	+ 2–4 days		

+ = positive reaction; acid and gas in carbohydrate broth. ± = acid only or acid and bubble of gas in carbohydrate broth; some strains motile, others nonmotile, others not tested. − = negative reaction; carbohydrate not fermented in 40 days. −0 = not tested.

Only one cultural group of *P. aerogenoides* was isolated in this study. This group is apparently the same as type 1721 of Stuart *et al.* (1943). Of the 16 cultures in this group, 13 were antigenically related, as shown by spot plate and tube agglutination tests. The 3 unrelated strains were all from hospital patients. The antigenic relationship of the other 13 strains was not a close one, however, since adsorption of the serum with the heterologous strains failed to reduce the titer for the homologous organism.

Two cultural groups of *P. intermedium* were found. Group 1 resemble most closely the type 1421 of Stuart *et al.* (1943), with the differences that all cultures studied produced hydrogen sulfide, 2 of the 12 strains produced acid and gas in salicin in 11 and 20 days, and all were definitely aerogenic with all carbohydrates fermented. Of this cultural type, 7 were isolated from hospital patients, 5 from normal Arabs, and none from normal Americans. Antigenically, 4 of the 7 strains which were tested against one antiserum were related. The 4 related strains were from normal Arabs, and the other 3 were from hospital patients. Adsorption experiments were not conducted. Group 2 strains were not held on citrate medium more than 1 week, and it is possible that longer incubation would have brought out citrate utilization. These cultures differed from group 1 in being hydrogen-sulfide-negative and negative or slow on sucrose. All organisms of this group were isolated from normal Americans.

Three cultural groups of *P. coliforme* were isolated. The 9 cultures of group 1 were negative on both lactose and sucrose after 3 weeks in 1 per cent and 40 days in 5 per cent concentrations of the sugars. Six of the 9 were positive in salicin after 2 to 4 days. Some of the cultures were also tested on xylose and inositol. Three of the cultures were negative on xylose, 5 produced acid and gas immediately, and 1 culture was positive after 3 days. Eight cultures tested on inositol were all negative. Antigenically, only 2 of the 9 cultures were related. Adsorption experiments were not done. Three cultures of this group were from normal Americans and 6 were from normal Arabs.

The 3 cultures of group 2 differed from those in group 1 in fermenting lactose in 9 to 35 days, 2 were negative on salicin, and 1 was positive after 6 days. Two of these cultures were from normal Arabs, and 1 culture was from an individual suffering from a mild diarrhea. The 8 cultures of group 3 were either negative on lactose or produced only acid, or acid and a bubble of gas. All 8 cultures were positive on sucrose. Four cultures of this group were from hospital patients; 3 were from normal Arabs and 1 was from a normal American.

PATHOGENICITY

Evidence for the pathogenicity of *Paracolobactrum* species in recent years has been slowly accumulating (Parr, 1939; Weil, 1943). Stuart and Rustigian (1943) presented strong evidence for the pathogenicity of one type of *P. aerogenoides*. Stuart *et al.* (1943) presented further direct evidence for the pathogenicity of *P. aerogenoides* and *P. coliforme* strains. Young (1946) reported three strains of *Paracolobactrum* organisms containing *Salmonella* XXXVIII antigen isolated from patients with clinical symptoms but not from asymptomatic persons.

The present communication adds further evidence for the pathogenicity of *Paracolobactrum* species. The isolation of these organisms from 60 per cent of gastroenteritis cases as compared to 20 per cent of healthy individuals living under similar (Army) sanitary conditions is at least indicative of pathogenicity, particularly since in only a negligible number of cases were they found in association with commonly accepted pathogens. Although objection may be made to attaching such significance to the distribution of these organisms, particularly in view of the relatively large number of isolations from healthy individuals, it will be observed that relatively large numbers of *Salmonella* and *Shigella* species were also isolated from healthy individuals. Furthermore, Neter (1943) has stated that persons with no history of bacillary dysentery may harbor dysentery bacilli. The writer in this investigation also isolated *Salmonella* cultures from at least one healthy person (himself) with no history of previous *Salmonella* infection. It would therefore seem quite possible to find pathogenic *Paracolobactrum* species in healthy persons not susceptible to their invasive capabilities.

It is of further interest, concerning relative distribution of enteric pathogens, that Stuart, Wheeler, and McGann (1946) reported that an antigenically heterogenous, anaerogenic paracolon organism was isolated almost exclusively from gastroenteritis patients in the vicinity of Providence, Rhode Island, with only 1 strain being isolated from over 300 normal individuals. This same type, however, was isolated frequently from normal individuals in the State of Florida.

Aside from the significance implied by the relative distribution of *Paracolobactrum* organisms (tables 1 and 2), additional evidence points to the pathogenicity of at least the *P. aerogenoides* type isolated in this study. In the majority of cases, colonies on the plates were not in pure culture and indeed in many cases were not even very abundant, probably because most specimens were obtained from 3 days to a week after onset of the symptoms. In two cases, however, specimens were obtained within a few hours after the onset of the symptoms. These cases were primarily surgical patients (appendectomy and amputation) suffering from mild diarrhea. Specimens from these patients yielded from few to numerous, apparently identical, colorless colonies on EMB and SS agars, and after streaking to SS agar from selenite-f broth. All cultures so isolated were *P. aerogenoides*, and no commonly accepted pathogens were found.

In another case, a member of the laboratory staff, who one evening had eaten dinner away from the organization's mess, suffered from a very mild diarrhea the next morning. His stool specimens showed the presence of *P. coliforme* of group 2, though not in very great abundance. No other pathogens were found. No additional evidence beyond the suggestive distribution was obtained for the pathogenicity of *P. intermedium*.

Biochemically, the *P. coliforme* cultures of group 1 are of particular interest. These organisms are apparently identical with the once-designated *Salmonella columbensis*, recently studied by Fulton (1943). This author studied 18 strains of this biochemical type, the majority of which were obtained from gastroenteritis patients. He felt that this organism, although not belonging to the genus *Salmonella*, was in fact worthy of not only specific but generic rank.

In the present study, the 9 organisms of this group were all obtained from apparently normal individuals and none from hospital cases. The outstanding characteristic of these organisms, however, was their complete lack of ability to ferment either 1 per cent or 5 per cent lactose and sucrose after 3 weeks' and 40 days' incubation in the respective concentrations. Fulton showed that his 18 strains failed to ferment 5 per cent lactose in 10 days. These reactions do indeed seem to set the organisms apart, and though under the proposed scheme of Borman *et al.* (1944) they are now included in the *P. coliforme* species, it seems possible that further information will show them to be better delineated as a separate species. It might be added, parenthetically, that of 28 indole-positive strains isolated both before and during this investigation which were negative on lactose and sucrose in 1 per cent concentrations after 1 to 3 weeks, 13 produced acid or acid and gas in 5 per cent of either one or both of the carbohydrates within 1 to 35 days.

Although the fact that the relatively small number of specimens examined in this investigation, together with the relatively small number of organisms of each cultural group isolated, makes it inadvisable to state dogmatically that the *Paracolobactrum* species studied are pathogenic, it is felt that the evidence presented warrants the conclusion that the *P. aerogenoides* type is very probably pathogenic in this area (Casablanca, North Africa) and that certain of the cultural groups of *P. intermedium* and *P. coliforme* are possibly pathogenic. It is felt further that more investigations of the nature herein reported should be made on a larger scale and preferably in the field, with especial attention to the incidence of *Paracolobactrum* species. Such studies should yield valuable information concerning the general pathogenicity of these organisms, as well as bring to light any geographical differences in the pathogenicity of the cultural types isolated.

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SUMMARY

A study of the comparative distribution of *Paracolobactrum*, *Salmonella*, and *Shigella* species in an area highly endemic for enteric infections showed that *Paracolobactrum* species were found much more frequently in cases of gastroenteritis than were either of the other two groups. The *Paracolobactrum* organisms were found with much greater frequency in hospital cases than in normal individuals or two groups—namely, normal Arabs and normal American troops. *Salmonella* and *Shigella* species were found with almost equal frequency in all three groups.

Study of the *Paracolobactrum* species yielded evidence that the one cultural group of *P. aerogenoides* isolated, consisting of 16 strains, 13 of which were antigenically related, was very probably pathogenic in this area. It was considered

possible that some of the *P. intermedium* and *P. coliforme* strains might be pathogenic.

The significance of the distribution of these organisms in normal individuals in this and other areas is discussed.

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CLASSIFICATION OF THE RICKETTSIAE OF ROCKY MOUNTAIN SPOTTED FEVER AND OF ENDEMIC (MURINE) TYPHUS

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An impediment to a logical classification of the rickettsiae pathogenic for man has been the use of the generic term *Dermacentroxenus* for the organism of Rocky Mountain spotted fever. This designation was applied by Wolbach (1919), the term being derived from the generic name of the arthropod host, *Dermacentor andersoni*. The rickettsia was named *Dermacentroxenus rickettsi*. The same generic term was employed by Wolbach and Todd (1920) to designate the organism seen in sections of the capillaries, arterioles, and veins of Mexican typhus fever subjects. This organism was named *Dermacentroxenus typhi*. The use of *Dermacentroxenus* as applied to the organism of endemic (murine) typhus has not been recognized, and the term *Rickettsia* is universally accepted as the proper generic name of the organism of this disease as it is closely related to that of epidemic typhus, *Rickettsia prowazekii* (type species).

The purpose of classification is to arrange organisms which possess genetic relationships in groups. Just what criteria are to be used in making such arrangements is often debatable. The partial localization of the rickettsiae of Rocky Mountain spotted fever in the nucleus of cells in Rocky Mountain spotted fever in contrast to the intracytoplasmic localization of typhus rickettsiae has been cited as justification for the separation of the organism of spotted fever from those of epidemic (European) typhus and endemic (murine) typhus (Pinkerton, 1936).

There are, on the other hand, many characteristics which relate the spotted fever organism to those of epidemic and endemic typhus. These organisms are similar morphologically; they have common antigenic factors with certain *Proteus* strains; they show slight resistance to heat and chemical agents; they are nonfilterable; they occur in the endothelial cells of the small blood vessels; they are all concerned in diseases of man characterized by fever and exanthema; and there is evidence of some immunological relationship, Castañeda and Silva (1941) having shown that recovered typhus-infected guinea pigs are markedly more resistant to highly virulent spotted fever strains than are normal guinea pigs to the same strain. All are transmitted to man by arthropods.

If *Dermacentroxenus* should be considered acceptable as the generic designation for the organism of Rocky Mountain spotted fever, then it follows that other organisms of the rickettsial group which might be as closely related to one another as is the organism of Rocky Mountain spotted fever to *Rickettsia prowazekii* would also fall in different genera, each of which would consist of only one or two species. It is well recognized that many other organisms designated as rickettsiae are much farther removed from the type species, *Rickettsia prowazekii*, than is the organism of Rocky Mountain spotted fever, and these might well be assigned to other genera.

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The elimination of the term *Dermacentroxenus* would clarify the situation and and permit of an orderly classification of the well-recognized rickettsiae, as well as of many of the other "rickettsiae" when further information concerning them is available. On this basis the rickettsia of Rocky Mountain spotted fever becomes *Rickettsia rickettsi* proposed by Brumpt (1922) in his *Précis de parasitologie*, the species designation being fully valid.

With reference to the species designation *typhi* for the organism of endemic typhus, this term has priority over all others, including *manchuriae* (Kodama *et al.*, 1931), *mooseri* (Monteiro, 1932), *exanthematofebri* (Kodama, 1932), *muricola* (Monteiro and Fonseca, 1932), *murina* (Megaw, 1935), *fletcheri* (Megaw, 1935), *prowazeki* var. *mooseri* (Pinkerton, 1942). As suggested by Philip (1943), the name *typhi* is valid transferred to the genus *Rickettsia* as *Rickettsia typhi* or *Rickettsia prowazekii* subsp. *typhi*. Recently acquired information on the serology of epidemic and endemic typhus (Plotz, 1943), as well as differences of growth capacity and differences in clinical manifestations in man and laboratory animals, would seem to justify differentiation on a species basis, and therefore *Rickettsia typhi* (Wolbach and Todd)[2] may be considered a suitable name for the organism of endemic (murine) typhus. Though Wolbach and Todd (1920) designated the disease which they were studying as "typhus exanthématique au Mexique," Steinhaus (1946) states that Dr. Wolbach, as stated in a personal communication, believes for various reasons that the disease studied was of the murine type. The creation of a new name, *Demacentroxenus typhi*, for the causative agent rather than the acceptance of the term *Rickettsia prowazekii*, the recognized agent of European typhus, for the organism seen by Wolbach and Todd lends support to this belief.

The species designation *manchuriae* antedates *mooseri*. In the April, 1932, number of the *Kitasato Arch. Exptl. Med.*, v. 9, p. 99, Kodama, Kono, and Takahashi in the paper "Demonstration of *Rickettsia manchuriae* appearing in the stomach epithelial cells of rat fleas and rat lice infected with so-called Manchurian typhus" refer to a previous paper entitled "On the experimental observation of the so-called Manchurian typhus and its etiological agent (*R. manchuriae*)" (*Saikingaku-Zasshi*, Japanese, no. 426, 427, Aug. and Sept., 1931). Though the latter paper is not available in this country, the contents of the paper in the April, 1932, issue of the *Kitasato Arch. Exptl. Med.* and the paper which follows it, which is probably an English translation of the paper in Japanese referred to, since it has the same title, afford sufficient evidence to justify acceptance of the identify of the disease the authors were investigating (Manchurian typhus) with endemic (murine) typhus and also the identity of the rickettsiae found in the scrapings of tunica of guinea pigs, rats, and rabbits with the so-called "Mooser bodies." Monteiro (1932) in a paper on typhus exanthematicus of São Paulo in *Memorias do instituto Butantan*, v. 6, p. 97, proposes the name *Rickettsiae mooseri* for the "Mooser bodies." The date of publication of this paper as well as of the entire volume was July, 1932 (see v. 6, p. 134, and v. 7, p. 367, of the *Memoirs*).

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[2] Although the species name *typhi* as used in the binomial *Dermacentroxenus typhi* Wolbach and Todd clearly has priority, the name *Rickettsia typhi* was proposed by Do Amaral and Monteiro (1932) in 1931 for the organism of the eastern type of Rocky Mountain spotted fever. However it has been shown that the eastern type of spotted fever does not differ from the western or Bitterroot type, and, since this designation for eastern spotted fever has not been recognized, it may be considered invalidated and therefore as not conflicting with the use of this binomial for the rickettsia of endemic (murine) typhus.

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PENICILLIN

X. THE EFFECT OF PHENYLACETIC ACID ON PENICILLIN PRODUCTION[1]

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As penicillin chemistry unfolded during the early days of production development, it soon became evident that there existed more than one compound having the pharmacological and bactericidal properties associated with the name "penicillin." American workers had isolated a pure crystalline penicillin (G) which was characterized by the benzyl radical and which yielded phenylacetic acid as a degradative product, whereas the crystalline penicillin isolated by the English workers (penicillin F) contained the Δ-2-pentenyl radical and yielded no phenylacetic acid on degradation, Δ-3-hexenoic acid being obtained in its place (Committee on Medical Research, Office of Scientific Research and Development, Washington; and the Medical Research Council, London). It was of interest, therefore, to ascertain the effect of adding phenylacetic acid to the culture medium in or upon which the mold was grown. Two possibilities were anticipated, both based on the assumption that the mold could use the phenylacetic acid as a building stone for the penicillin molecule. If this supposition were true, it would follow that (a) yields would be increased if synthesis of phenylacetic acid by the mold were the bottleneck in its penicillin production, and (b) the penicillin G:penicillin F ratio would be increased.

The experiments described below show that the total penicillin yield was increased in both surface and submerged cultures, but no marked change in the ratio of penicillin types could be demonstrated as being due to phenylacetic acid.[4]

METHODS AND MATERIALS

All of the surface production cultures were grown at 24 C, in 200-ml pyrex Erlenmeyer flasks containing 50 ml of medium. The submerged cultures were also grown at 24 C, but in 300-ml Erlenmeyer flasks containing 125 ml of medium and shaken on a Ross-Kershaw machine.

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[4] The effect of phenylacetic acid on penicillin production was communicated in a restricted monthly report, no. 16, November 20, 1943, to Dr. A. N. Richards, Chairman, Committee on Medical Research, Office Scientific Research and Development, who in turn sent copies to all penicillin producers and to many research groups in this country and abroad. The phenomenon here described has been generally applied industrially. Owing to the strategic significance of penicillin, publication of this research has been delayed.

The assays were conducted by the cylinder plate method of Abraham *et al.* (1941), as modified by Schmidt and Moyer (1944). Some assays were made by the same procedure, except that *Bacillus subtilis* NRRL B-558 and *Staphylococcus aureus* NRRL B-313 were used in parallel as the test organisms. Schmidt *et al.* (1945) demonstrated that a comparison of assay values obtained with *S. aureus* and *B. subtilis* gives an indication of the type of penicillin present.

The inoculum for the submerged production cultures consisted of a suspension of tiny pellets, about 1 mm in diameter, which were obtained after 3 days' growth in the following medium: lactose monohydrate, 40.0 g; glucose monohydrate, 4.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KH_2PO_4, 0.50 g; $NaNO_3$, 3.0 g; $ZnSO_4 \cdot 7H_2O$, 0.080 g; corn steep liquor, 36.0 g; and distilled water to make 1 liter. Portions of 125 ml of this medium were dispensed in 300-ml Erlenmeyer flasks. One gram of sterile, dry $CaCO_3$ was added to each flask just before inoculation. Inoculations were made with 10-ml portions of a heavy suspension of ungerminated spores. These cultures were incubated at 24 C on the Ross-Kershaw shaking machine.

The production media contained the corn steep liquor and lactose which have been shown by the authors (1946) to be essential to good penicillin yields in both surface and submerged cultures.

The experimental error of the cup plate assay on crude culture filtrates was frequently of sufficient magnitude under routine conditions to render difficult a very accurate evaluation of units per ml based on the dual assays. The variation in the assays appeared to be greater with the crude penicillin filtrates than with highly purified samples of the F and G types of penicillin.

Three fungus strains, *Penicillium notatum* NRRL 1249.B21 (Moyer and Coghill 1946*a*), *P. notatum* NRRL 832 (Moyer and Coghill 1946*b*), and *Penicillium chrysogenum* NRRL 1951.B25 (Raper and Alexander, 1945) were employed in these investigations.

EXPERIMENTAL RESULTS

Surface cultures. Concentrations of phenylacetic acid varying from 0.025 to 0.8 g per liter, added at the time of inoculation, were first employed in surface cultures of *P. notatum* NRRL 1249.B21. On the second day a concentration of phenylacetic acid of 0.2 g per liter had caused a definite inhibition of growth; at 0.4 g per liter there was a marked toxicity; and at 0.8 g per liter growth was completely inhibited. As the cultures aged and the pH increased, the toxicity of phenylacetic acid appeared to decrease. There was some increase in penicillin yields apparently due to phenylacetic acid. The cultures containing 0.2 g per liter of phenylacetic acid, although slower in growth and pH change, gave as high yields of penicillin as the cultures containing only 0.025 g of phenylacetic acid per liter. This result suggested that higher yields of penicillin might be obtained if the toxic effect of such concentration of phenylacetic acid were eliminated during the stage of early growth.

The first attempt to eliminate such toxicity was made by adding the phenylacetic acid to 2-day-old surface cultures. Under these conditions a marked increase in penicillin yields was obtained by the addition of 0.1 to 0.6 g of phenylacetic acid per liter of medium. The tolerance for this compound was much greater when it was added to 2-day-old cultures than when it was added at the time of inoculation with ungerminated spores. This difference in phenylacetic acid tolerance was due either to a greater resistance of the 2-day-old mycelium or to a close relationship between toxicity and the pH of the medium, similar to that known to exist for acetic and benzoic acids.

To test the effect of adding phenylacetic acid at different ages of the culture, three series of surface cultures were prepared. To reduce the toxic effect associated with low pH, the initial pH of the medium was raised from 4.0 to 4.6 by adding KOH. Phenylacetic acid was then added to one series of cultures at the time of inoculation, and to another series when the cultures were 2 days old. At the initial pH of 4.6, it was found that 0.4 g per liter of phenylacetic acid was just slightly toxic, as shown by a slower growth and pH change than occurred in the control cultures (table 1). The presence of phenylacetic acid in both culture series caused a marked increase in the penicillin yield. The assay values based on *B. subtilis* were not significantly different from those based on *S. aureus*, regardless of phenylacetic acid, indicating that the penicillin was of the G type even in the control cultures of *P. notatum* NRRL 1249.B21.

Since these results showed that some advantage was gained by raising the initial pH of the medium from 4.0 to 4.6, another series of cultures was prepared with the initial pH values at 4.2, 4.7, 5.2, and 5.8, with and without phenylacetic acid (0.30 g per liter) added at the time of inoculation with ungerminated spores. At an initial pH of 4.2, moderate toxicity of phenylacetic acid was again encountered (table 2). With the initial pH at 5.2 and 5.8, there was no indication of toxicity due to the phenylacetic acid. A penicillin yield of 266 units per ml was obtained in those cultures containing phenylacetic acid with an initial pH of 5.2 or 5.8.

The best penicillin yields with phenylacetic acid were obtained in a medium containing a special steep liquor nutrient prepared commercially by a starch manufacturing company. The initial pH was adjusted to 5.6 with NaOH, and 0.40 g per liter of phenylacetic acid was sterilized in the basal medium. Under these conditions, a penicillin yield of 316 units per ml was obtained in 7 days (table 3). The superiority of this special steep liquor nutrient over the ordinary corn steep liquor has been demonstrated by comparative tests in other surface culture experiments.

Submerged-shaker cultures. The effect of phenylacetic acid on penicillin yield was studied concurrently in both surface and submerged cultures. In the submerged cultures of *P. notatum* NRRL 832, the potency value in terms of units obtained from *B. subtilis* assays was 20 to 30 per cent lower than from *S. aureus* assays. This difference in assay values was believed to be due to the presence of penicillin F as the predominant type. Hence, attention was directed not only to the possible increase in total penicillin yield but also to possible changes in the type of penicillin found in these submerged cultures.

The submerged-shaker production flasks were inoculated with small portions of a preformed pellet type growth of *P. notatum* NRRL 832. The initial pH

TABLE 1

Effect of phenylacetic acid (0.40 g per liter) on penicillin yields, added at time of inoculation and after 2 days to surface cultures of P. notatum NRRL 1249.B21

PHENYLACETIC ACID ADDITIONS	CULTURE AGE, DAYS				
	3	4	5	6	7
Penicillin, units per ml (*S. aureus* NRRL B-313)					
None	47	106	126	143	130
At start	36	90	160	191	194
At 2 days	50	128	170	189	178
Penicillin, units per ml (*B. subtilis* NRRL B-558)					
None	—	111	126	150	132
At start	—	96	164	191	200
At 2 days	—	134	176	200	196
pH of filtrates					
None	6.3	7.1	7.5	7.7	8.0
At start	5.4	6.5	7.2	7.5	7.9
At 2 days	6.0	6.8	7.5	7.7	8.1
Dry weight of fungus growth, g per culture					
None	0.73	0.97	1.11	1.20	1.17
At start	0.41	0.81	0.98	1.18	1.23
At 2 days	0.67	0.96	1.10	1.23	1.19

Culture medium: Lactose monohydrate, 55.0 g; glucose monohydrate, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KH_2PO_4, 0.50 g; $NaNO_3$, 3.0 g; $ZnSO_4 \cdot 7H_2O$, 0.022 g; corn steep liquor, 90.0 g; and distilled water to make 1 liter.

Initial pH: 4.6.

TABLE 2

Effect of initial pH of medium on the value of phenylacetic acid in penicillin production by P. notatum NRRL 1249.B21 in surface cultures

INITIAL pH	PHENYLACETIC ACID PER LITER	CULTURE AGE, DAYS				
		3	4	5	6	7
Penicillin, units per ml (*S. aureus* NRRL B-313)						
	g					
4.2	0.0	45	78	102	130	159
4.2	0.3	21	67	109	138	188
4.7	0.0	54	85	125	150	194
4.7	0.3	47	106	177	200	224
5.2	0.0	45	80	127	157	178
5.2	0.3	48	106	159	200	266
5.8	0.0	40	80	123	150	178
5.8	0.3	51	106	168	200	266

Culture medium: Lactose monohydrate, 44.0 g; glucose monohydrate, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KH_2PO_4, 0.50 g; $NaNO_3$, 3.0 g; $ZnSO_4 \cdot 7H_2O$, 0.022 g; corn steep liquor, 78.0 g; and distilled water to make 1 liter. KOH used to make pH adjustments.

of the production medium was 4.2. In the preliminary experiments, phenylacetic acid was added at the time of inoculation and to 1-day-old production cultures. Under these conditions, phenylacetic acid at 0.4, 0.8, and 1.2 g per liter resulted in no increase in penicillin yield. There was a marked toxicity with phenylacetic acid at 0.6 and 1.2 g per liter.

It was known that the pH of these production cultures rose rapidly, and it seemed likely that, as in the surface cultures, the toxicity of phenylacetic acid would be less if additions were made to 2- or 3-day-old cultures. A series of cultures was prepared in which phenylacetic acid (0.8 g per liter) was added to 1-, 2-, and 3-day-old production cultures (table 4). Phenylacetic acid added to the 1-day-old cultures was quite toxic, inhibiting both growth and penicillin production, but additions to the 2- or 3-day cultures showed no toxicity and a significant increase in penicillin yield as compared with the control cultures. On the third day, 0.40 g of $CaCO_3$ was added to one of the control cultures. As a result of this treatment, a rapid growth occurred and a good yield of penicillin was obtained on the seventh day. This was evidence that the inhibitory or toxic effect of phenylacetic acid could largely be overcome by raising the initial pH of the medium or by making the addition to 2- or 3-day-old

TABLE 3

*Effect of phenylacetic acid on penicillin yields by P. notatum NRRL 1249.B21 in surface cultures using a special corn steep liquor nutrient**

PHENYLACETIC ACID PER LITER	CULTURE AGE, DAYS			
	4	5	6	7
	Penicillin, units per ml (*S. aureus*)			
g				
0.0	98	146	189	194
0.4	132	233	263	313
	Penicillin, units per ml (*B. subtilis*)			
0.0	—	160	192	189
0.4	—	222	250	316
	pH of filtrates			
0.0	7.2	7.5	7.7	7.9
0.4	7.2	7.6	7.7	7.9
	Dry weight of fungus growth, g per culture			
0.0	0.70	0.89	0.92	0.88
0.4	0.68	0.85	0.90	0.89

Culture medium: Same as given in table 2, except that 70.0 g per liter of special steep liquor nutrient was employed instead of ordinary corn steep liquor. The initial pH of the medium was adjusted to 5.6 with NaOH.

* Special corn steep liquor nutrient 14 Ea supplied by a commercial firm.

cultures. The comparative assay values with *B. subtilis* and *S. aureus* at 6 and 7 days did not indicate any change in type of penicillin produced, although the total yield of penicillin had been increased by the addition of phenylacetic acid.

In one culture series the initial pH was raised from 4.1 to 5.0 by the addition of KOH. After 1-day incubation, the pH of these cultures had arisen to 6.6 in the case in which 0.8 g of phenylacetic acid per liter had been added to the cultures. There was no evidence of toxicity due to the phenylacetic acid, and there was a significant increase in the penicillin yield (table 5). Again assays with both *S. aureus* and *B. subtilis* showed that phenylacetic acid, although increasing the total yield of penicillin, did not suggest a change in the type of penicillin produced. Other experiments showed there was no advantage in raising the initial pH of the medium much above 5.2.

TABLE 4

Effect of 0.8 g per liter of phenylacetic acid on penicillin yields by P. notatum NRRL 832, when added to 1-, 2-, and 3-day-old submerged production cultures

PHENYLACETIC ACID PER LITER	CULTURE AGE, DAYS					
	2	3	4	5	6	
	Penicillin, units per ml					
g						
Control	11	22	36	57	65, (51)	68, (51)
1-day add'n	2	3	4	4	4	—
1-day add'n.*	2	3	8	30	59	95, (88)
2-day add'n	15	35	50	71	80, (69)	85, (76)
3-day add'n	14	32	45	71	85, (72)	86, (63)
	pH of filtrates					
Control	7.1	7.7	7.3	7.2	7.9	8.1
1-day add'n	4.4	4.7	4.9	4.9	5.2	—
1-day add'n.*	4.4	4.7	6.0	7.6	7.8	7.4
2-day add'n	7.2	7.5	7.4	7.4	7.8	7.9
3-day add'n	7.2	7.3	7.4	7.3	7.9	8.0

Culture medium: Amounts per 1 liter: Lactose monohydrate, 30.0 g; $NaNO_3$, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.125 g; KH_2PO_4, 0.250 g; $ZnSO_4 \cdot 7H_2O$, 0.022 g; corn steep liquor, 50.0 g.

All cultures inoculated with 4 ml of a 3-day-old pellet suspension.

Initial pH: 4.2.

Assays values in parentheses determined with *B. subtilis* NRRL B-558; all others with *S. aureus*.

* 0.40 g $CaCO_3$ added per culture on third day.

Several investigations were made to determine the concentration of phenylacetic acid required to give the maximum penicillin yield. It was not possible to determine clearly such an optimum concentration of phenylacetic acid, possibly because of some uncontrolled factors, such as activity or uniformity of the inoculum, foaming, variations in shaker speed, etc., which varied to some extent from week to week. A comparison of 0.2 g and 0.8 g of phenylacetic acid per liter (added at 30 hours) on penicillin yield is shown in table 6.

TABLE 5

The effect of phenylacetic acid when added to 1-day-old submerged production cultures of P. notatum NRRL 832 for which the initial pH was raised to 6.0 with KOH

PHENYLACETIC ACID PER LITER	CULTURE AGE, DAYS					
	2	3	4	5	6	7
	Penicillin units per ml					
0.00	22	48	76, (54)	80	65	58
0.80	26	60	90, (69)	100	95	80
	pH of filtrates					
0.00	8.0	7.6	7.6	8.0	8.2	8.3
0.80	7.9	7.6	7.7	8.0	8.2	8.3

Culture medium: Amounts per 1 liter: Lactose monohydrate, 30.0 g; $NaNO_3$, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.125 g; KH_2PO_4, 0.250 g; $ZnSO_4 \cdot 7H_2O$, 0.022 g; corn steep liquor, 50.0 g.

Cultures inoculated with 6 ml of a 3-day-old pellet inoculum.

Assay values in parentheses determined with *B. subtilis*, NRRL B-558; all others with *S. aureus*.

TABLE 6

Effect of phenylacetic acid on penicillin yields by P. notatum NRRL 832, when added to 30-hour-old, pellet-inoculated submerged production cultures

PHENYLACETIC ACID PER LITER	CULTURE AGE, DAYS				
	2	3	4	5	6
	Penicillin, units per ml				
g					
0.00	11	23	50	84, (66)	95, (75)
0.20	18	36	80, (66)	102, (88)	125, (105)
0.80	12	34	83, (68)	113, (101)	141, (120)
	pH of filtrates				
0.00	7.7	7.6	7.3	7.5	7.9
0.20	7.7	7.6	7.3	7.4	7.8
0.80	7.6	7.6	7.3	7.4	7.8

Culture conditions same as given in table 4.

Cultures inoculated with 6 ml of a 3-day-old pellet suspension.

At 30 hours the pH was 6.3.

Assays values in parentheses determined with *B. subtilis* NRRL B-558; all others with *S. aureus*.

There was no significant difference in the pH change between the control cultures and those receiving phenylacetic acid. These and other experiments showed that a maximum increase in penicillin yield could be attained by supplying phenylacetic acid at 0.2 to 0.8 g per liter. No further increase in penicillin yield was ever obtained by increasing the concentration of phenylacetic acid beyond 0.8 g per liter of medium.

Part of the results of the foregoing experiment is presented in figure 1. At 30 hours the pH had risen from 4.1 to 6.3 when 0.8 g per liter of phenylacetic acid was added. During the 6-day incubation period, 5-ml samples were removed once daily for assay and pH determinations. The amount of total growth was based on a visual score. After 4 days no increase in the amount of fungus growth could be detected. After 3 days there was a marked increase in the formation of a yellow pigment which diffused from the pellet growth out into the medium. The peculiar type of pH curve has already been discussed by the authors (1946*b*).

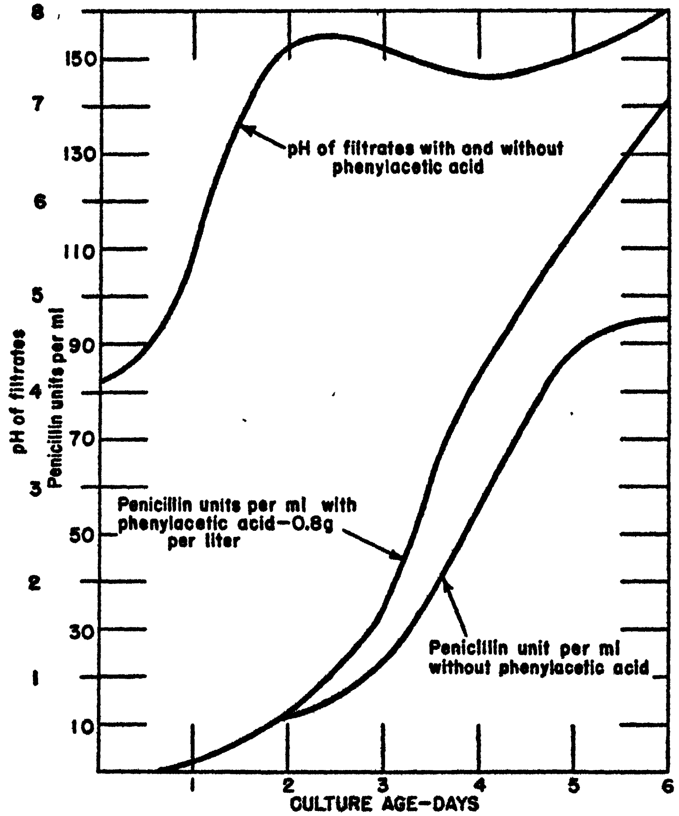

FIG. 1. EFFECT OF PHENYLACETIC ACID ON GROWTH RESPONSES AND TOTAL PENICILLIN YIELD (ASSAY WITH S. AUREUS) BY P. NOTATUM NRRL 832, WHEN ADDED TO 30-HOUR-OLD PELLET-INOCULATED PRODUCTION CULTURES

A strain of *P. chrysogenum* NRRL 1951.B25 was employed in a series of cultures in which the initial pH was raised to 5.3 by the addition of $CaCO_3$ or to pH 5.6 with NaOH. Phenylacetic acid (0.4 g per liter) was added to the nutrient medium before sterilization. These cultures were inoculated with a suspension of ungerminated spores. In the presence of phenylacetic acid, approximately the same levels of penicillin were produced whether NaOH or $CaCO_3$ was used as the neutralizing agent. It is worth noting that less penicillin was produced in the absence of phenylacetic acid when NaOH was employed as the neutralizing agent than when $CaCO_3$ was used. At the fifth day, penicillin yields with *S. aureus* were 106 units in both NaOH and $CaCO_3$ cultures (table 7). Again no effect of phenylacetic acid on the type of penicillin produced could be shown by the differential assays.

TABLE 7

Effect of phenylacetic acid, 0.4 g per liter, on penicillin production by P. chrysogenum NRRL 1951.B25 in submerged cultures

INITIAL pH OF MEDIA	PHENYLACETIC ACID PER LITER	CULTURE AGE, DAYS				
		3	4	5	6	7
		Penicillin units per ml				
	g					
NaOH to pH 5.6	0.0	26	40, (29)	60, (48)	68, (52)	54, (41)
	0.4	36	75, (69)	106, (78)	102, (87)	80, (69)
$CaCO_3$* pH 5.3	0.0	53	80, (63)	95, (82)	100, (84)	68, (46)
	0.4	50	85, (73)	106, (80)	106, (71)	71, (54)
		pH of filtrates				
NaOH to pH 5.6	0.0	8.0	8.1	7.7	8.0	8.2
	0.4	8.0	7.9	8.0	8.4	8.3
$CaCO_3$ pH 5.3	0.0	8.0	8.1	7.9	8.4	8.5
	0.4	8.0	8.1	7.9	8.3	8.6

Culture medium: Lactose monohydrate, 27.5 g; glucose monohydrate, 5.5 g; $MgSO_4 \cdot 7H_2O$, 0.125 g; KH_2PO_4, 0.25 g; $ZnSO_4 \cdot 7H_2O$, 0.022 g; corn steep liquor, 50.0 g; and distilled water to make 1 liter.

All cultures inoculated with ungerminated spores.

Phenylacetic acid added before medium sterilization.

Assay values in parentheses determined with *B. subtilis* NRRL B-558; all others with *S. aureus*.

* $CaCO_3$ 5.0 g per liter, added to cool, sterile medium.

Sodium benzoate, used at 0.8 g per liter in both surface and submerged culture, caused no increase in penicillin yield, nor was there any indication of a change in type of penicillin produced.

Various concentrations of phenylacetic acid were employed in both surface and submerged cultures grown on a synthetic medium. In no case was it possible to demonstrate clearly an increase in total penicillin yield or a change in type of penicillin due to the effect of phenylacetic acid.

The addition of small portions of a finely ground wheat bran (about 50 per cent starch) and phenylacetic acid to submerged cultures of *P. chrysogenum* 1951.B25 with a nonpellet type of inoculum resulted in a marked increase in the speed of penicillin accumulation (table 8). The particles of bran seemed to act as focal points for the germinated spores, resulting in soft "fuzzy" pellets. The addition of wheat bran in the presence of phenylacetic acid caused not only an increase in total penicillin yield but also caused a change in the assay ratio which might be interpreted as indicating greater accumulation of penicillin G over that obtained in the absence of bran. This effect of bran in combination with phenylacetic acid has been repeated in many experiments. The pH of the cultures receiving 5.0 to 10.0 g of wheat bran per culture was always lower up to the fourth day than the pH observed in the control cultures. After the fourth day there was usually little difference in pH between cultures with or without bran. In a large number of experiments, penicillin assays by use of *S. aureus* and *B. subtilis* were nearly identical up to 3 days in cultures receiving only phenylacetic acid, if the pH was not higher than 7.6. Identical results could not be obtained by adding wheat flour in an amount equivalent to that occurring in the bran or by the addition of bran in the absence of phenylacetic acid.

TABLE 8

Effect of finely ground wheat bran on total yield and type of penicillin produced in submerged culture of P. chrysogenum 1951.B25

	CULTURE AGE, DAYS		
	3	4	5
Penicillin, units per ml			
Control	34 (32)	59 (41)	66 (47)
+ 0.5 g bran per culture	79 (84)	89 (96)	95 (95)
pH of filtrates			
Control	7.4	7.7	7.8
+ 0.5 g bran per culture	6.5	7.6	7.8
Bulk of growth, score			
Control	3.2	3.5	3.6
+ 0.5 g bran per culture	4.7	4.7	4.7

Culture medium: Amounts per 1 liter—corn steep liquor, 36 0 g; lactose monohydrate, 27.5 g; $MgSO_4 \cdot 7H_2O$, 0.125 g; KH_2PO_4, 0.25 g; $NaNO_3$, 1.50 g; $ZnSO_4 \cdot 7H_2O$, 0.088 g; phenylacetic acid, 0.30 g.

Culture size: 200 ml in 1-liter Erlenmeyer flasks. Bran and phenylacetic acid sterilized in presence of medium; 1.2 g sterile $CaCO_3$ added per culture just before inoculation with a nonpellet type of inoculum (10 ml per culture). Culture incubated at 25 C on a rocking cradle.

Assay values in parentheses determined with *B. subtilis* NRRL B-558; all others with *S. aureus*.

DISCUSSION

The effectiveness of phenylacetic acid in increasing penicillin yields in both surface and submerged cultures is closely related to its toxicity. The degree of this toxicity depends both on the concentration of the phenylacetic acid and the pH of the culture medium. The toxicity of phenylacetic acid is believed to be due to the undissociated molecule, as has been demonstrated with respect to acetic acid by other investigators. Kiesel (1913) observed that formic and acetic acids were more toxic for *Aspergillus niger* than were mineral acids. He attributed the toxicity of these organic acids to the undissociated molecule. Cruess and Irish (1932) found that *P. glaucum* could tolerate 160 times more acetic acid at pH 7.0 than at pH 2.5. Kirby *et al.* (1937) showed that the toxicity for *A. niger* at a fixed concentration was a function of the pH of the medium. At pH 5.5 to 6.0 acetic acid slightly inhibited germination, but it had only a slight effect, ranging from slight inhibition to a slight stimulation, on the ultimate growth of several molds. It was claimed that the undissociated molecule, not the acetate ion, was responsible for toxicity. The increased antiseptic efficiency of weak acids has been proved in the majority of cases to be due to the undissociated acid molecules. Such was found to be true for acetic, propionic, butyric, choroacetic, bromopropionic, oxalic, selenious, nitrous, benzoic, salicylic, sulfurous, and hypochlorous acids, and also for phenol (Huntington and Rahn, 1945). Therefore, it is believed that the toxicity of phenylacetic acid is probably due to the undissociated molecule.

A steady rise in the pH of the medium made it possible to add phenylacetic acid in effective amounts to 2-day-old surface cultures without serious toxicity. It was found to be a more convenient and equally effective procedure to raise the initial pH of the medium to 5.0 to 5.8, and to sterilize the phenylacetic acid in the culture medium. Using a pellet inoculum in the submerged cultures, better penicillin yields were obtained by adding the phenylacetic acid during the fermentation, when the pH had risen to approximately 5.5, than were obtained by adjusting the initial pH to 5.5. When ungerminated spores were used for inoculum, the toxicity of phenylacetic acid could be avoided by adjusting the initial pH to a suitable level by means of $CaCO_3$ or a soluble alkali.

The quantitative relationship between the amount of phenylacetic acid added and the increase in penicillin yield was not always apparent in these experiments. Approximately 5 mg of phenylacetic acid, as based on the data in tables 2 and 3, gave a 1-mg increase in the penicillin yield. A slight increase in penicillin yield was obtained with 0.025 g of phenylacetic acid per liter. If all the phenylacetic acid added (tables 2 and 3) was utilized directly in penicillin synthesis, then the penicillin yield should have been several times that actually obtained. Thus it appears that factors other than the amount of phenylacetic acid limit the amount of penicillin that accumulates in the fermented liquor. The optimal amount of phenylacetic acid over a wide range of pH has not been systematically determined; however, best penicillin yields were obtained in the surface cultures with phenylacetic acid at 0.3 to 0.4 g per liter and an initial pH of 5.0 to 5.8.

The role of whole-wheat bran in bringing about a marked increase in the rate of penicillin accumulation is not clearly understood. The fungus growth surrounded the bran particles to form soft, "fuzzy" pellets. This bran contained about 50 per cent starch, but the addition of an equivalent amount of starch or aqueous bran extracts had little, if any, effect on penicillin yields. The bran cultures gave a much more rapid growth than the control cultures and a near maximum yield of penicillin was obtained before the medium became very alkaline. The apparent change in the type of penicillin was encountered only upon addition of bran to cultures containing the phenylacetic acid. It seems likely that a difference in the stability of the various types of penicillin at the various pH levels in the culture medium would have as much effect on the assay ratio as might result from alteration in the proportion of types of penicillin actually produced.

Phenylacetic acid has been regarded as a "building block" in the synthesis of penicillin G. Thus phenylacetic acid would not be effective in the synthesis of the F and K types which do not contain the benzenoid ring structure. At the time these investigations were made, all differential assay ratios were interpreted in terms of penicillins F and G only. It is now generally known that penicillin X is present in fairly large amounts in surface cultures and to some extent in submerged cultures. Penicillin X gives a high ratio in the differential assay. Since no quantitative separations of the various types of penicillin were made during the course of these investigations, all deductions as to penicillins present were originally based on the differential assay (Schmidt *et al.*, 1945) with *S. aureus* and *B. subtilis.* No significant change in type G penicillin produced in culture could be directly attributed to phenylacetic acid with the possible exception of those cultures containing wheat bran. In both surface and submerged cultures there was a pronounced increase in the total penicillin yield due to the addition of phenylacetic acid to the culture medium. The failure, normally, to show a change in the proportions of the types of penicillin produced in the presence of phenylacetic acid may be due to the high concentrations of corn steep liquor employed, to certain inadequacies of the differential assay, and to possible differences in stability of the penicillins at various pH levels in the culture medium.

SUMMARY

Marked increases in total penicillin yield were obtained in surface cultures of *Penicillium notatum* NRRL 1249.B21 and in submerged cultures of *P. notatum* NRRL 832 and *Penicillium chrysogenum* 1951.B25 by the addition of phenylacetic acid.

The toxicity of phenylacetic acid was closely associated with the initial acidity of the culture medium. This toxicity could be sufficiently overcome by raising the pH prior to inoculation or by adding the phenylacetic acid during the fermentation after the pH of the medium has risen above the critical level.

Only in the presence of whole-wheat bran in submerged culture was it possible to obtain evidence that phenylacetic acid had an effect on the type of penicillin produced.

The optimum concentration of phenylacetic acid was not clearly determined, but it appears to be between 0.2 g and 0.8 g per liter of culture medium.

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THE INFLUENCE OF TEMPERATURE UPON OPSONIZATION AND PHAGOCYTOSIS[1]

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The phagocytic reaction is complex, separable into three fundamental stages: (1) sensitization of the particle by serum components, (2) ingestion of the sensitized particle by the phagocyte, and (3) disposal of the ingested particle. The unqualified term "phagocytosis" has come to refer largely to the process of ingestion without definite consideration either of events preceding this second step or of those following it. It is not our intention to criticize this practice, but to emphasize the complexity of the phenomenon of phagocytosis and its importance in the analysis of experimental results. The present study attempts to separate the first two stages of the reaction, i.e., sensitization of the organisms and actual ingestion, each process being subjected separately to temperature alterations.

Most reports in the literature have shown that opsonization at 37 C is more effective than at lower temperatures. Bulloch and Atkin (1905), however, found that opsonin was lost from fresh normal serum after incubation with *Staphylococcus albus* for 10 minutes at either 0 C or 37 C. An experiment by Dean (1905) suggested that *Staphylococcus aureus* was ingested ten times as readily following incubation with heated normal serum for 30 minutes at 37 C as following a similar incubation at 6 C or 8 C. Ledingham (1908) observed the amount of ingestion of *Staphylococcus aureus* following sensitization at 37 C to be greater than that following sensitization at 0, 7, or 18 C. Sellards (1908) likewise claimed that opsonization at 37 C led to more phagocytosis than opsonization at 0 C for 15 minutes.

The studies to be summarized in this paper have attempted a reinvestigation of temperature influences, employing a different temperature range and rotation of phagocytic systems, a technique introduced since the earlier studies.

METHODS

In each of the experiments, exudative polymorphonuclear leucocytes were obtained from the peritoneal cavity of a guinea pig previously injected with a sterile irritant, and fresh normal serum was recovered by cardiac puncture of the same animal. The general procedure followed in the collection and preparation of these materials was the same as that described in a previous report (Harmon, Zarafonetis, and Clark, 1946).

The test organism was *Staphylococcus aureus*, a viable suspension in saline, prepared every 6 weeks. The bacteria and serum (0.1 ml of each) were incu-

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bated at the desired temperature for a period of 30 minutes, leucocytes (0.1 ml) were then added and the complete system was incubated 5 minutes more. The terms "sensitization" and "opsonization" will be used throughout to refer to the incubation of serum and organisms before the addition of phagocytes. The term "phagocytosis" will denote incubation of the complete system, the leucocytes being added after the sensitization period. Preparations were made from 0.02 ml of the mixture placed on each of several slides; the films were dried quickly, stained with methylene blue, and examined. In order to eliminate any element of personal bias in examination, descriptive labeling on each slide was covered with adhesive tape and an arbitrary number assigned to each slide. Estimation of the extent of phagocytosis was accomplished by recording the percentage of leucocytes taking part in the reaction (Hamburger, 1912). A rough phagocytic index (Leishman, 1902) was also obtained by noting the percentage of leucocytes which ingested more than 5 bacteria. Since these two determinations and that of a strict phagocytic index appeared to exhibit the same trends with variations in temperatures, the determinations included in final analysis have been limited to the total percentage of phagocytes active. Two hundred cells were counted on each slide, and two slides were examined for each set of conditions studied, thus making a total of 400 cells for each determination.

The first series (experiments 21 through 30) was designed to study the effect of increasing temperature from 37 to 40 C. Two portions of bacteria were sensitized at 37 C and then subjected to phagocytosis at 37 and 40 C, respectively; two other portions of bacterial cells were sensitized at 40 C and phagocytized at 37 and 40 C, respectively. The period of opsonization was 30 minutes, that of phagocytosis was 5 minutes. In this series, sensitization was effected without rotation of the bacteria and serum, but during incubation of the complete system, the tubes were rotated mechanically end-over-end at the rate of 2.5 revolutions per minute.

In the second series (experiments 31 through 36), we employed temperatures 22 and 37 C, with methods similar to those of the previous experiments except that sensitization also was carried out in a rotating tube rather than in a stationary one. Controls to determine the relative amounts of phagocytosis within 5 minutes at 22 and at 37 C were included in experiments 32 through 36; no previous period for sensitization was employed in these controls.

RESULTS

Comparisons have been made on the basis of the total percentage of phagocytes active under the conditions described. In order to compare two sets of conditions, the total percentage of phagocytes active at the lower temperature of opsonization or phagocytosis was divided into the total percentage of phagocytes active at the higher temperature. Thus a figure of comparison greater than 1.00 indicates that increased temperature was favorable to opsonization or phagocytosis, as the case may be; a figure less than 1.00 indicates that increased temperature inhibited opsonization or phagocytosis. The results are summarized in table 1. As judged from the average comparative figures at 37 and at 40 C, the higher temperature favored opsonization, regardless of the temperature of phagocytosis. However, these mean averages are somewhat misleading.

TABLE 1

The effect of temperature changes upon opsonization and phagocytosis of Staphylococcus aureus

Effect of Increased Temperature upon

EXPT. NO.	OPSONIZATION*		PHAGOCYTOSIS†	
	$\frac{40\text{-}37\ C}{37\text{-}37\ C}$	$\frac{40\text{-}40\ C}{37\text{-}40\ C}$	$\frac{37\text{-}40\ C}{37\text{-}37\ C}$	$\frac{40\text{-}40\ C}{40\text{-}37\ C}$
21	1.10	1.39	0.91	1.15
22	1.70	0.86	1.94	0.98
23	1.04	1.43	0.96	1.31
24	0.93	0.91	1.03	1.02
25	0.89	1.03	1.00	1.17
26	0.97	1.03	1.13	1.19
27	1.01	0.83	1.43	1.14
28	1.30	1.23	1.37	1.29
29	1.12	1.26	1.06	1.19
30	0.93	1.16	1.01	1.27
Average...............	1.10	1.11	1.18	1.17
	$\frac{37\text{-}22\ C}{22\text{-}22\ C}$	$\frac{37\text{-}37\ C}{22\text{-}37\ C}$	$\frac{22\text{-}37\ C}{22\text{-}22\ C}$	$\frac{37\text{-}37\ C}{37\text{-}22\ C}$
31	0.91	1.00	1.52	1.67
32	0.73	0.57	1.72	1.34
33	0.78	0.68	1.49	1.29
34	0.77	0.69	1.39	1.33
35	0.48	0.73	0.97	1.49
36	0.61	0.94	1.18	1.78
Average...............	0.71	0.77	1.38	1.48

* Values obtained by dividing total per cent of phagocytes active after opsonization of bacteria at the higher temperature by total per cent of phagocytes active after opsonization of bacteria at the lower temperature—phagocytosis temperature constant, opsonization temperature varied—e.g., $\frac{40\text{–}37\ C}{37\text{–}37\ C}$ first figure that of opsonization temperature, second that of phagocytosis temperature.

† Values obtained by dividing total per cent of active phagocytes following phagocytosis at the higher temperature by total per cent of active phagocytes at the lower temperature—opsonization temperature constant, phagocytosis temperature varied—e.g., $\frac{37\text{–}40\ C}{37\text{–}37\ C}$ first figure that of opsonization temperature, second that of phagocytosis temperature.

In experiments with phagocytosis at 37 C (table 1) only 6 of 10 tests showed greater phagocytosis when opsonization was effected at the higher temperature, and in 2 of these (experiments 23 and 27), the increases were insignificant;

experiment 22 was responsible for the higher average. When phagocytosis was conducted at 40 C, opsonization at the higher temperature was more effective in 7 of 10 tests; but here, again, the increases in 2 experiments (experiments 25 and 26) were so slight as to be negligible. In this case (phagocytosis at 40 C), however, the average value is more significant since the extremes are not so marked.

With 22 and 37 C, opsonization at the lower temperature was definitely more effective than at the higher (table 1) whether subsequent phagocytosis was conducted at 22 or 37 C. Opsonization at 37 C followed by phagocytosis at 37 C in experiment 31 was the same as that at 22 C, but this was the only instance in 12 comparisons (phagocytosis at 22 and at 37 C).

The effect of temperature increases upon ingestion of bacteria already sensitized was more marked. In experiments in which 37 and 40 C were compared, 16 of the 20 determinations showed more phagocytosis at 40 C than at 37 C. Two of the aberrant results occurred when preliminary sensitization had been at 37 C (experiments 21 and 23), one with opsonization at 40 C (experiment 22), and one showed exactly the same result at the two phagocytic temperatures (experiment 25, opsonization at 37 C). However, the increased values obtained in three of the remaining tests were small (experiments 24 and 30 with opsonization at 37 C, and experiment 24 with opsonization at 40 C). On the basis of many checks of our results we have considered only values of 1.05 and greater as significant. That the effect of temperature increases over this range (37 to 40 C) was more marked and more consistent upon phagocytosis than upon opsonization can be seen by a comparison of the "opsonization" and "phagocytosis" columns in table 1. The greatest single effect of temperature increase was that upon phagocytosis as the temperature was raised from 22 to 37 C.

TABLE 2

The effect of previous opsonization of Staphylococcus aureus upon the extent of subsequent phagocytosis

Relative Amount of Phagocytic Activity when Preceded by Opsonization

EXPT. NO.	TEMPERATURES OF OPSONIZATION-PHAGOCYTOSIS COMPARED WITH PHAGOCYTOSIS ALONE*			
	$\frac{22\text{-}22\text{ C}}{22\text{ C}}$	$\frac{37\text{-}22\text{ C}}{22\text{ C}}$	$\frac{22\text{-}37\text{ C}}{37\text{ C}}$	$\frac{37\text{-}37\text{ C}}{37\text{ C}}$
32	1.99	1.45	1.06	0.60
33	1.20	0.95	0.80	0.54
34	1.44	1.04	0.78	0.54
35	1.60	0.77	0.71	0.52
36	1.75	1.06	0.89	0.82
Average..............	1.59	1.05	0.85	0.60

* Values obtained by dividing total per cent of phagocytes active following both opsonization and phagocytosis at the indicated temperatures by that following phagocytosis alone at the temperature indicated, e.g., $\frac{22\text{-}22\text{ C}}{22\text{ C}}$

In only 1 of 12 comparisons was phagocytosis less at 37 C than at 22 C; this exception followed sensitization at 22 C (experiment 35).

After the observation made in experiment 31 that opsonization was less effective at 37 C than at 22 C, some doubt arose in our minds as to whether preliminary incubation of bacteria and serum enhanced subsequent phagocytosis at all. Therefore, in experiments 32 through 36 we included a control consisting of bacteria, serum, and leucocytes mixed only during the period allowed for phagocytosis (i.e., 5 minutes). Relative amounts of phagocytic activity occurring in these tubes as compared with those subjected to 30 minutes' preliminary sensitization of the bacteria are considered in table 2. Figures of comparison were obtained in the same manner as in table 1, but for table 2 the total percentage of active phagocytes following both opsonization and phagocytosis at the indicated temperatures was divided by that following phagocytosis with no previous opsonization at the corresponding temperature.

Except when both opsonization and phagocytosis were studied at 22 C, the preliminary period failed to increase substantially the final phagocytic activity. The average amount of phagocytosis at 22 C was somewhat greater with preliminary opsonization at 37 C, but this occurred in only 3 of 5 determinations and 2 of these tests (experiments 34 and 36) did not show marked differences. Five minutes of phagocytosis at 37 C was more effective than phagocytosis following 30 minutes' opsonization at either 22 C or 37 C; only 1 of 10 determinations (experiment 32, sensitization at 22 C) disagreed with this trend.

DISCUSSION

In the 37 to 40 C series, opsonization at the higher temperature (30 minutes' stationary incubation) tended to be more effective than that at 37 C as determined by the extent of subsequent phagocytosis. When 22 and 37 C were compared (tubes rotated during 30 minutes of incubation), however, the increase in temperature consistently decreased the amount of opsonization. Such findings are in disagreement with those of Dean and of Ledingham, for both these investigators reported greater phagocytosis of *Staphylococcus aureus* following preliminary incubation of the bacteria with serum at 37 C than at lower temperatures. The data of table 1 suggest that the preparation of bacteria for ingestion proceeds better at 22 C than at 37 C, but the results given in table 2 indicate that more phagocytosis occurs at 37 C without any previous incubation of bacteria and serum.

In view of these findings, it seems likely that there is some inhibitory reaction proceeding during the preliminary incubation of bacteria and serum, thus adding a fourth factor to be considered in "phagocytosis." What may be the nature of such a reaction cannot be stated, but the incubation of viable organisms in the presence of cell-free, fresh, normal serum is perhaps important. It is doubtful that such serum is sufficiently inhibitory to the organism to prevent all metabolic activity, and the fact that this hypothetical inhibitory reaction occurs more readily at 37 C than at 22 C, but apparently no better at 40 C than at 37 C, further suggests the metabolic nature of the phenomenon.

The possible toxic effects of viable organism on phagocyte may be in these investigations part of the answer. Since a coagulase-positive strain of *Staphylococcus aureus* was employed in these studies, the question of coagulase as inhibitory agent must be raised. Hale and Smith (1945) observed that certain strains of *Staphylococcus aureus* were able to coagulate guinea pig plasma, and, as a result, the organisms were ingested less readily by polymorphonuclear leucocytes; our strain had this characteristic. This may have been a factor in our experiments, for the blood recovered for serum was centrifuged as rapidly as possible after collecting, and plasma may have been present in sufficient quantity for the coagulase reaction. We have not attempted to determine the effects of temperature and time of opsonization upon coagulase activity in these systems and can, therefore, offer this only as a possibility with regard to the inhibitory mechanism. The study of other organisms in such systems would be of value in a continuation of these investigations, as well as the effect of temperature variation and other factors upon the phagocytic cell itself.

Hale and Smith (1945) mentioned also that there was a brief delay during which no apparent inhibition of phagocytosis by coagulase-positive strains occurred. This point may be important, since we have shown in an earlier paper that ingestion increases regularly with elevation of temperature over the range of 22 to 42 C, when mixtures were rotated for ten minutes without previous incubation of bacteria with normal serum. In that series of experiments it was noted that the average amount of phagocytic activity following rotation of guinea pig phagocytic systems with *Staphylococcus aureus* at 37 C was over twice as much as that which followed incubation at 22 C. The highest figure observed in the present studies did not approach this, and the average increase was even less However, in the simple phagocytic tests without previous sensitization included in experiments 32 to 36, a figure similar to that of the earlier experiments was obtained when phagocytosis was compared at 22 and at 37.

The influence of temperature changes upon phagocytosis (opsonization temperatures constant) following preliminary opsonization at any given temperature is considerably more clear-cut. This was true of the 37 to 40 C series and even more so in the 22 to 37 C group. This effect, in addition to being more consistent, was quantitatively more marked than the corresponding effect upon opsonization.

It should be stressed that fresh normal serum has been employed in all of these investigations; the role of complement has not, therefore, been separately studied. Tests employing immune serum should lead to more clear-cut separation of the relation of the thermolabile and the thermostable serum fractions, those which act specifically as well as those which are nonspecific.

SUMMARY AND CONCLUSIONS

In phagocytic systems composed of guinea pig exudative leucocytes, fresh, normal guinea pig serum, and *Staphylococcus aureus*, previous sensitization of the organisms at 40 C tended slightly toward a greater amount of ingestion at either 37 or 40 C than did opsonization at 37 C. Phagocytosis at 40 C following preliminary opsonization of bacteria at either 37 or 40 C resulted in more phagocytic activity than at 37 C.

With 22 and 37 C as test temperatures, opsonization (as measured by subsequent phagocytosis) was impaired by the higher temperature but phagocytosis was enhanced by it. Phagocytosis at 37 C was decreased by any preliminary incubation of viable organisms and fresh normal serum. Such inhibition was more marked when preliminary incubation occurred at 37 C than at 22 C.

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RELATION BETWEEN THE RATE OF GROWTH OF A MUTANT STRAIN OF ESCHERICHIA COLI AND THE EFFICIENCY OF ITS UTILIZATION OF ARGININE

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A mutant strain of *Escherichia coli* that requires the addition of arginine for growth was isolated in this laboratory by Roepke *et. al.* (1944) following serial transfer of the parent culture in a complete medium. This strain (1572-228)[2] is unable to utilize citrulline or ornithine for growth even in the presence of limiting concentrations of arginine.[3] During studies on the determination of arginine by the use of this mutant strain, we observed that the final turbidity reached by the organism with limiting concentrations of arginine was decreased by the addition of an arginine-free mixture of amino acids and growth factors or of a yeast extract preparation which had been freed of arginine by alkaline hydrolysis. This effect of supplements was similar to that noted in experiments with mutant 532-171, which requires the addition of methionine for growth (Lampen *et al.*, 1947).

This report deals with the conditions under which the decreased final turbidity is observed and the effect of this phenomenon on the assay of arginine in protein hydrolyzates.

EXPERIMENTAL

The basal medium (glucose, asparagine, and salts) and the general methods employed have been reported previously (Lampen *et al.*, 1947). Arginine-low natural materials were prepared by alkaline hydrolysis. This treatment converts arginine to citrulline and ornithine (Fox, 1938). Ten-gram samples of Difco yeast extract or of Difco peptone were dissolved in 100 ml of 5 N NaOH and autoclaved at 15 pounds' pressure for 1 hour and for 3 hours. The hydrolyzates were neutralized with 10 N H_2SO_4 and diluted to 200 ml. Each ml was then equivalent to 50 mg of the original material. The yeast extract had contained 0.16 per cent arginine (by assay with the mutant strain) before hydrolysis and the peptone 3.9 per cent. After 1 hour of hydrolysis the yeast extract did not support growth of the mutant at concentrations to 50 mg per 10 ml ($<$ 0.02 per cent arginine) whereas the peptone still contained 0.2 per cent arginine after 3 hours. A yeast extract sample hydrolyzed 1 hour was employed in the experiments described here.

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[2] This strain is available from the American Type Culture Collection, Georgetown University, Washington, D. C., as no. 9661.

[3] Lampen, J. O., and Jones, M. J., unpublished data.

The extent of growth was determined with a photronreflectometer (Libby, 1941). With our instrument one unit of galvanometer deflection is equivalent to 13.4 million cells of *Escherichia coli* per ml.

RESULTS

The effect of the addition of the arginine-low yeast extract preparation on the response of the mutant to arginine is illustrated by figure 1. The final turbidity with limiting concentrations of arginine was about 30 per cent less in the presence of the supplement. The maximum growth obtained with excess arginine was generally increased slightly. The latter effect may be due to the buffering action of the yeast extract preparation. Similar results were obtained when an arginine-free mixture of amino acids and growth factors (as used by Roepke *et al.*, 1944) was added in place of the yeast extract preparation.

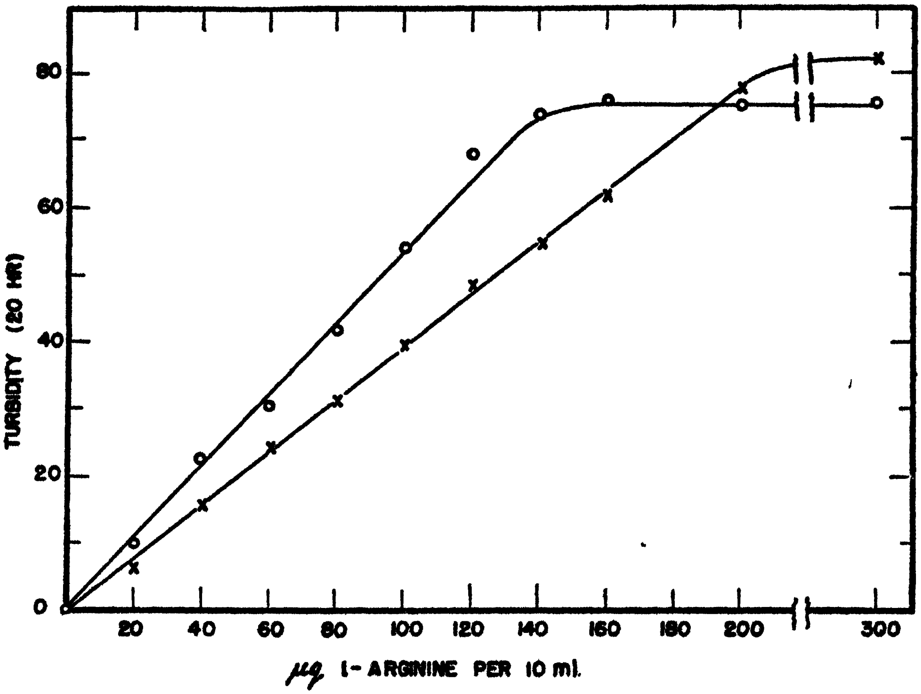

FIG. 1. THE RESPONSE OF MUTANT 1572-228 TO *l*-ARGININE

○ Basal medium.

× Basal medium plus 25 mg per 10 ml of an alkaline hydrolyzate of yeast extract.

When protein hydrolyzates were assayed using the unsupplemented basal medium, the apparent arginine content of the samples decreased as the test concentration was increased. This effect is probably due to the depressing effect of the other amino acids present on the response of the mutant to arginine. The values obtained under these conditions (table 1) were considerably lower than those reported in the literature. In the presence of the alkaline hydrolyzate of the yeast extract the effect of the amino acids of the test sample was no longer significant. The apparent arginine content of the test samples increased and the values were constant at varying concentrations of the test sample. With a concentration of 20 to 30 mg of the yeast preparation per 10 ml, the values obtained were comparable to those in the literature (table 1). Recoveries of added arginine have ranged from 96 to 112 per cent in several experiments using 25 mg of the yeast preparation per 10 ml. The Na_2SO_4 concentration under these conditions was 0.0625 M. Neither the final turbidity nor growth rate of

TABLE 1

Effect of an alkaline hydrolyzate of yeast extract on the apparent arginine content of proteins

PROTEIN*	ARGININE CONTENT %†					VALUES FROM LITERATURE %
	Alkaline hydrolyzate of yeast extract mg per 10 ml					
	None	10	20	25	30	
Ovalbumin‡	4.45	5.2	5.6	5.6, 5.7	5.8	5.6,§ 5.66‖
Horse hemoglobin‡ (once recrystallized)	2.85	3.2		3.6		3.5,§ 3.59‖
Casein Hammarsten (Merck)	3.0–3.3 (3.15)¶	3.3		3.45–3.8 (3.65)¶		

* All samples were hydrolyzed by autoclaving for 10 hours at 120 C with 10 per cent HCl in a sealed tube.

† All values are based on measurements of turbidity after 20 to 24 hours of incubation.

‡ These samples were obtained through the generosity of Dr. E. E. Snell and were those analyzed by McMahan and Snell (1944).

§ McMahan and Snell (1944).

‖ Vickery (1940).

¶ Average of 4 experiments.

TABLE 2

Relation between logarithmic phase growth rate and final response of mutant 1572-228 to arginine

l-ARGININE	ALKALINE HYDROLYZATE OF YEAST EXTRACT	VELOCITY CONSTANT (*K*)*	TURBIDITY READING AT 24 HOURS
μg per 10 ml	mg per 10 ml		
50	0	0.83	34
50	25	1.36	25
100	0	0.90	67
100	25	1.39	47
200	0	0.89	96
200	25	1.39	101

* $K = \frac{2.303}{T_2 - T_1} \log N_2/N_1$ where N = millions of cells per ml; T = hours of incubation.

the mutant on the basal medium containing limiting concentrations of arginine was significantly affected by the addition of this concentration of Na_2SO_4.

The relation between the logarithmic phase growth rate and the final response of the mutant to arginine in shown in table 2. The addition of the alkaline hydrolyzate of yeast extract increased the logarithmic phase velocity constant 54 to 63 per cent, but reduced significantly the final response of the mutant to limiting concentrations of arginine. With excess arginine, approximately the same maximal turbidity was reached in this experiment in the presence and absence of the yeast preparation. The lag period was unchanged (ca. 2 hours) by the addition of the hydrolyzed yeast. There is then an inverse relation between the rate of growth and the final growth obtained with limiting concentrations of arginine. The effect of supplementation on the response of the mutant to arginine appears analogous to that observed with a methionine-requiring strain of *Escherichia coli* (Lampen *et al.*, 1947).

SUMMARY

The final response of an arginine-requiring mutant strain (1572-228) of *Escherichia coli* to limiting concentrations of arginine is reduced by the addition of an arginine-free mixture of amino acids and growth factors or of an alkaline hydrolyzate of yeast extract. This reduction in final response occurs under conditions in which the logarithmic phase growth rate is increased. Thus there appears to be an inverse relation between the growth rate and the efficiency of arginine utilization by the mutant strain.

In the presence of the yeast preparation the values obtained for the arginine content of protein hydrolyzates are in agreement with those obtained by the use of other procedures.

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MICROMONOSPORIN, AN ANTIBIOTIC SUBSTANCE FROM A LITTLE-KNOWN GROUP OF MICROORGANISMS[1]

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The genus *Micromonospora* belongs to one of the least-known genera of the *Actinomycetales* (Waksman and Henrici, 1943). It comprises both mesophilic and thermophilic forms and is found in soils, in manure and composts, in lake bottoms, and in other natural substrates. The micromonosporae are characterized by the formation of single spores on side branches. Several species have now been recognized.

In a survey of the distribution of antagonistic properties among the actinomycetes (Waksman, Horning, Welsch, and Woodruff, 1942), a strain of *Micromonospora* was found to produce an antibiotic substance that was active against various gram-positive bacteria. This organism possessed rather interesting physiological properties. It grew in stationary liquid media or on solid media only to a very limited extent, producing an orange to brown slimy growth, with a smooth and shiny surface, which later turned brown or almost black. However, in an aerated submerged condition, this organism grew very abundantly in the form of orange-colored compact clumps of "colonies" which nearly filled the culture flask; the medium itself remained clear.

The organism grew well on glucose and starch media, the latter being rapidly transformed to reducing sugar, which accumulated in the medium. Organic sources of nitrogen were preferred to the inorganic forms. The organism was, therefore, grown in a starch tryptone medium in a submerged state. Under these conditions, the organism hydrolyzed all the starch (900 mg dry raw starch per 100 ml of medium) and produced, in 5 days, 250 mg cell material on a dry basis; about 225 to 250 mg of reducing sugar was accumulated. The pH of the medium usually became alkaline in glucose media and acid in starch media.

Five- to seven-day-old culture filtrates gave an activity of 1,000 to 3,000 dilution units against *Bacillus subtilis*, but only 100 units against *Staphylococcus aureus* and *Sarcina lutea*; gram-negative bacteria were not affected. The results of a typical experiment are brought out in table 1. When the culture filtrate was removed and fresh medium added, a considerable amount of activity was again produced. The nature of the replacement medium did not seem to have any appreciable effect, since even distilled water gave nearly as high activity as the complete medium.

The antibacterial substance produced in the medium did not dialyze through a collodion membrane; it was adsorbed by norit, but could be removed therefrom either by aqueous buffer solutions or by common organic solvents.

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[1] Journal Series paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

The active substance was not stable when the pH was adjusted to less than 3.0 or to greater than 9.0; about two-thirds of the activity was lost in 1 hour when the filtrate was heated at 100 C. Pepsin and trypsin were without effect on its activity.

TABLE 1

Influence of carbon source on the growth of Micromonospora sp. and on the production of micromonosporin

CARBON SOURCE	NITROGEN SOURCE	INCUBATION DAYS	GLUCOSE MG PER 100 ML OF MEDIUM*	GROWTH MG	UNITS OF ACTIVITY		
					B. subtilis	*B. mycoides*	pH
Glucose	Tryptone	4	820	55	>300	100	
Starch	Tryptone	4	190	178	>300	25	
Starch	$NaNO_3$	4	0	Trace	30	0	
Glucose	Tryptone	6	745	85	>1,000	100	
Starch	Tryptone	6	197	277	100	30	
Starch	$NaNO_3$	6	22	57	<10	10	
Glucose	Tryptone	9	715	98	500	30	7.7
Starch	Tryptone	9	135	351	150	10	5.6
Glucose	Tryptone	13	685	134	300	30	8.0
Starch	Tryptone	13	62	356	>300	30	6.1

* Original medium contained 920 mg reducing sugar.

TABLE 2

The isolation and activity of micromonosporin

PREPARATION	VOLUME OR WEIGHT	ACTIVITY IN DILUTION UNITS PER MG OR PER ML			
		S. aureus	*B. mycoides*	*B. subtilis*	*S. lutea*
Culture filtrate	1,500 ml	25	20	300	300
Filtrate from $(NH_4)_2SO_4$, ppt.	—	<100	<100	<100	<100
Final dialyzed solution	75 ml	200	100	3,000	1,000
Residue after lyophilizing	78 mg	30,000	80,000	800,000	300,000
Acetone extract of mycelium	100 ml	3,000	1,000	30,000	10,000
Residue from acetone extract	168 mg	200,000	200,000	20,000,000	6,000,000

The antibiotic could be precipitated from the filtered culture medium by saturation with ammonium sulfate. The chocolate-brown precipitate was removed on filter cel, washed with a saturated solution of ammonium sulfate, and dissolved in 75 ml of a 5 per cent solution of sodium chloride. This solution was dialyzed for 1 day against running tap water and for 2 days more against distilled water, and finally lyophilized. The dry residue now dissolved only with difficulty in 5 per cent sodium chloride. The product gave a very strong positive test for carbohydrate by the Molisch reaction, the phloroglucinol, orcinol, and naphthoresorcinol reactions being negative; it contained 6.7 per cent nitrogen, and on hydrolysis with 0.5 ml of boiling 6 N hydrochloric acid for 16 hours, it gave 5.5 per cent amino nitrogen.

The mycelium removed by filtration from this culture was treated with acetone. The acetone extract was of a bright orange color. On adding an alcoholic ferric chloride solution, there was no color change. The orange color of the acetone extract was changed to red on adding dilute potassium hydroxide. Sodium hydrosulfite bleached the color to yellowish, and the extract oxidized hydriodic acid to iodine. Würster reagent was not affected. The antibiotic activity of the different fractions is given in table 2.

A more detailed chemical analysis of the preparation obtained from the culture filtrates showed it to be a highly pigmented and very unstable protein, associated with a carbohydrate, which has the solubility of an albumin. The preparation obtained from the mycelium by extraction with organic solvents appears to be a pigment with some of the characteristics of a quinone. The bacteriostatic spectra of the two substances are much alike. Neither is effective against gram-negative bacteria, such as *Escherichia coli.* Both show their greatest action against *B. subtilis,* with progressively less action against *S. lutea,* and least activity against *S. aureus* and *Bacillus mycoides.* As a working hypothesis, one may suggest that the organism secretes a pigment in combination with a protein, whereas the mycelium contains the free pigment.

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NOTES

CHANGES INDUCED IN THE NONSPECIFIC ANTIGENS OF SALMONELLA[1]

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Edwards and Moran (Proc. Soc. Exptl. Biol. Med., **61**, **242**) referred to the changes produced in *Salmonella* H antigens *in vitro* by growth in agglutinating serums. The present note deals with hitherto undescribed changes brought about in the nonspecific phases of Andrewes.

S. newport (VI, VIII: e, h-1, 2, 3 . . .) was changed to a form indistinguishable from *S. pueris* (VI, VIII: e, h-1, 2 . . .) by growth in serums which contained agglutinins for phase 1 (e, h) and for single factor 3. *S. thompson* (VI, VII: k-1, 5 . . .) was transformed into a culture serologically identical with *S. cardiff* (VI, VII: k-1, 10 . . .) by growth in serums for phase 1 (k) and single factor 5. *S. panama* (IX, XII: l, v . . . -1, 5 . . .) was changed to a form resembling *S. italiana* (IX, XII: l, v . . . -1, 11 . . .) by growth in serum containing agglutinins for phase 1 (l, v . . .) and for single factor 5.

The cultures to be changed were placed in semisolid medium to which appropriate serums were added. The serums immobilized the cultures until changes in the antigens of phase 2 allowed that phase to spread through the medium. The changed cultures were diphasic.

In addition 1, 2 . . . phases were obtained from the natural 1, 2, 3 . . . phases of *S. oregon* and *S. vejle;* 1, 10 . . . phases were derived from the 1, 5 . . . phases of *S. kottbus*, *S. javiana*, *S. zanzibar*, *S. uganda*, and *S. solt*; and a 1, 11 . . . phase was obtained from the 1, 5 . . . phase of *S. shangani*. It was shown previously by Kauffmann (personal communication) that the 1, 5 . . . phases which yielded 1, 10 . . . differed serologically from those which gave rise to 1, 11 . . . .

It should be emphasized that the changes produced in the organisms mentioned above in no way invalidate the Kauffmann-White classification, which is used for diagnostic and epidemiological purposes. It is thought that studies in induced variation may yield valuable information on the origin of the numerous serological types which occur in nature.

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[1] The investigation reported in this paper is connected with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director. It was supported in part by a research grant from the U. S. Public Health Service.

THE USE OF FRAGMENTED MYCELIAL INOCULUM IN THE CULTURE OF FUNGI[1]

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Savage and Vander Brook (J. Bact., 52, 385) have recently reported the use of fragmented mycelia of *Penicillium notatum* NRRL 832 and *Penicillium chrysogenum* NRRL 1951 as inocula in the production of penicillin. They found that mycelium minced in a Waring "blendor" and diluted as much as 1:40,000 times adequately substituted for untreated mycelium used at a 1:10 dilution.

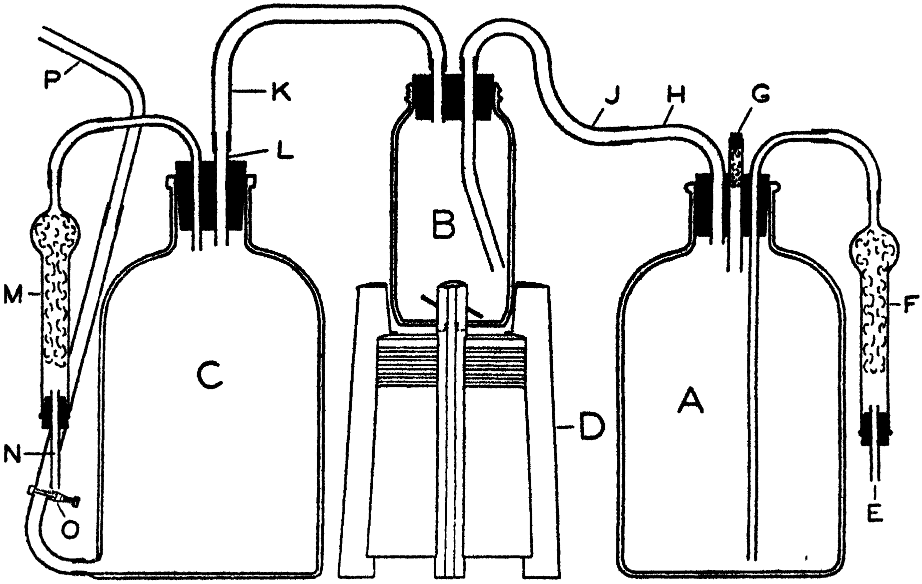

FIG. 1. APPARATUS FOR ASEPTIC MINCING AND TRANSFER OF MYCELIAL INOCULA

A. Four-L pyrex culture bottle. B. Duraglass fruit jar mounted on Waring "blendor." C. Pyrex aspirator bottle. The culture is grown in bottle A, air being introduced at E through filter F. Tube H is wrapped with cotton. The remainder of the apparatus is sterilized with tubes J, K, L, and P disconnected and wrapped with cotton. After assembly as shown, tube E is disconnected from the air line, tube N attached to an aspirator, clamp O closed, and jar B half filled by inverting bottle A. After this sample is minced, the suspension is drained into bottle C by inverting jar B. The operation may then be repeated. Tube K is then clamped and disconnected. The minced inoculum can be drained and dispensed through tube P.

We wish to report our experience in the use of a similar technique with other species of fungi and the development of simple equipment for handling the minced mycelium aseptically. Material so prepared has been found satisfactory for use in shaker flask experiments and as inoculum for large batches of media.

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[1] From studies conducted at Camp Detrick, Frederick, Maryland, from May, 1945, to August, 1946.

[2] Formerly Captain, CWS, and 1st Lt., CWS, respectively.

When a substantial quantity of minced mycelium was required, the equipment shown in figure 1 was employed. The mincer consisted of a Waring "blendor" on which the normal vessel was replaced by a one-quart "Duraglass" fruit jar cut to hold the blending blades. This jar was connected to a culture bottle containing a well-developed mycelial culture of the desired organism. The mycelium was passed into the blender jar in 200- to 300-ml lots, minced one minute, and passed out into a dispensing vessel. In this way substantial quantities of mycelial inoculum could be prepared aseptically in a short time.

The advantages of minced mycelial inoculum over spore inoculum are several. In laboratory studies employing liquid media, better agreement between replicates is obtained, the lag period in initiation of growth is shortened, and it is possible to work with asporogenous species or strains, or with organisms that sporulate poorly. In mass liquid cultures of *Helminthosporium oryzae* Breda de Haan the replacement of spore inoculum by 1 per cent minced inoculum resulted in a twofold increase in the yield of mycelium in two-thirds the time. Fragmented mycelium may well find a place as inoculum in industrial fermentations using fungi.

Minced mycelium has similarly been found suitable for shaker flask experiments and respiration studies. In this case the mincing apparatus can be smaller and has consisted of a small stainless steel cup fitting on the "blendor" base. It differed from that described by Hoffstadt and Tripi (J. Bact., **50**, 675) only in having a detachable base.

By mincing for one minute 50 ml of a 5- to 6-day-old mycelial culture of *Gibberella zeae* (Schw.) Petch grown in a shaker flask, approximately 1 to 1.5 × 10^6 mycelial fragments per ml were obtained. The fragments were freed of media by centrifuging and washing, using aseptic techniques. When examined microscopically, they were found to consist of 3- to 10-cell units, wholly viable and capable of germination at many points. Inoculation with 2 per cent of such material gave maximum mycelium production. This is far more than that found adequate by Savage and Vander Brook, but their report indicates that their culture was much heavier than ours.

THE USE OF A POLYVALENT ANTISERUM FOR PRELIMINARY IDENTIFICATION OF SALMONELLA CULTURES[1]

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A mixture of *Salmonella* antiserums was used by Felsenfeld and Young (J. Lab. Clin. Med., **30**, 550) and others in selecting cultures for serological typing. The first polyvalent anti-*Salmonella* serum to be made by injecting a single rabbit with all the known *Salmonella* antigens was prepared (by Bruner) and used by Ewing and Bruner (Med. Bull., Mediterranean Theater of Operations, **3**, 155). This antiserum was employed in Italy by the Section of Bacteriology, 15th Medical General Laboratory, in the examination of over 24,000 feces specimens from patients and food handlers.

After isolation on Kligler's iron agar (modified by addition of sucrose), all cultures were tested for their ability to hydrolyze urea. Those which did not attack this substance were tested with polyvalent antiserum. Bacteria which agglutinated were examined immediately by *Salmonella* typing methods.

All cultures were tested for fermentation of glucose, lactose, sucrose, and salicin; and for hydrogen sulfide and indole production. The media were held for 2 weeks or until acid production from one or more of the carbohydrates (lactose, sucrose, and salicin) or indole formation eliminated nonmembers of the genus *Salmonella*.

Unfortunately, data were not available for all the cultures isolated. Only those records with complete results of tests in polyvalent antiserum and biochemical reactions were used to compile the figures that follow: a total of 2,634 isolates. Of these, 546 proved to be *Salmonella* serotypes which agglutinated in polyvalent antiserum and gave characteristic biochemical reactions. There were 2,088 paracolon, *Proteus*, etc., cultures, 183 of which reacted in the polyvalent antiserum. These were eliminated subsequently from the genus *Salmonella* by failure to type in complete antigenic analysis and to conform in biochemical tests. The 1,905 isolates which failed to agglutinate in the polyvalent antiserum were excluded through biochemical nonconformity.

No cultures which failed to react in polyvalent antiserum were later proved to be *Salmonella* microorganisms. Twenty-two isolates (not included in the figures above) were not eliminated by biochemical tests within a 3-week incubation. These were examined for individual *Salmonella* antigens and found to have no serorelation to that group.

Shigella paradysenteriae serotypes Flexner I (V) and II (W) usually agglutinate in the polyvalent anti-*Salmonella* serum because of a relationship between the antigens of these serotypes and antigens XIII, XXII, and XXIII of the Kauffmann-White schema (authors' unpublished data).

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[1] Based on data obtained in the Section of Bacteriology, 15th Medical General Laboratory, United States Army.

These findings demonstrate the efficacy of the polyvalent *Salmonella* antiserum both for the early recognition of *Salmonella* cultures and the exclusion of nonmembers of the genus.

STUDY OF THE UTILIZATION OF SOME ORGANIC ACIDS BY ESCHERICHIA AND AEROBACTER

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The present communication reports the results of an investigation of the utilization of organic acids by members of the *Escherichia* and *Aerobacter* genera, when such acids are the sole source of organic material in the cultural media. The action of these bacteria on a larger group of organic acids than has been reported upon by other investigators (Koser: J. Bact., **11**, 409; Koser and Rettger: J. Infectious Diseases, **24**, 301; Ayers and Rupp: Abstracts Bact., **2**, 11; Braun and Cahn-Brooner: Biochem. Z., **131**, 226) is presented.

The medium used throughout this investigation had the following composition:

Sodium ammonium hydrogen phosphate	1.5 g
Potassium dihydrogen phosphate	1.0 g
Magnesium sulfate	0.2 g
Organic acid	1.0 g
Water	1,000 ml

Adjust to a pH value of 7 with normal sodium hydroxide.

Thirty-six organisms isolated from waters in all parts of the United States were used to inoculate the different media. These organisms were all members of either the genus *Escherichia* or the genus *Aerobacter*, most of which gave typical Voges-Proskauer, methyl red, and sodium citrate reactions, criteria which classified them definitely as belonging to these genera.

After inoculation, the media were incubated at 37 C for 72 hours. Readings were made at the end of 12, 24, 48, and 72 hours, growth being indicated by clouding of the medium.

The following acids when used as the sole source of carbon in media failed to support growth for any of the cultures of *Escherichia* and *Aerobacter*: *o*-aminobenzene sulfonic, *dl*-α-amino-*n*-butyric, *dl*-α-aminophenylacetic, *p*-aminophenylacetic, anthraquinone-β-sulfonic, benzidinedisulfonic, benzilic, β-benzoyl acrylic, *m*-bromobenzoic, *o*-bromobenzoic, *p*-bromobenzoic, α-bromo-*n*-caproic, *d*-camphoric, isocaproic, *m*-chlorobenzoic, cyanuric, α-β-dibromobutyric, 3,5-dinitrobenzoic, 3,5-dinitrosalicylic, 3,5-diiodo-4-hydroxybenzoic, diphenic, *m*-iodobenzoic, *o*-iodobenzoic, *p*-iodobenzoic, *m*-nitrobenzoic, resorcylic, and salicylic.

Succinic, lactic, malic, and *l*-aspartic acids supported growth for all of the 36 organisms from both genera of bacteria.

Propionic acid was found to support no growth for *Aerobacter* cultures, but 11 of the 18 members of the *Escherichia* organisms showed growth. Of the 7 organisms which did not show growth, 3 gave irregular tests with uric acid (uric-acid-positive) and 1 gave an irregular test with citric acid.

Butyric acid supported no growth for cultures of *Aerobacter*, but showed growth for all but 6 members of *Escherichia*. Of these 6 cultures, 5 were irregular in their reactions with either citric or uric acid.

Acetic acid supported growth for all but 3 of the *Escherichia* cultures and 2 of the *Aerobacter* cultures; mucic and phenylacetic for nearly all of both genera; and malonic for most of the *Aerobacter* and several of the *Escherichia* organisms. Benzoic acid gave a slight growth with several of the *Aerobacter* and 1 of the *Escherichia* organisms. β-Phenyl-α-β-dibromopropionic gave a slight growth with several of the organisms of both types, and benzyl isothiourea and α-aminoisobutyric hydrochloride supported growth for some members of each genus; 4-amino-1,3-dimethylbenzene acetate also supported growth for several cultures of each genus.

Barbituric acid was positive with all but 1 culture of the organisms tested, and dichlorobarbituric and dibromobarbituric were positive for only a few cultures of both types of organisms.

THE PREPARATION OF SILICIC ACID JELLIES FOR THE CULTIVATION OF MICROORGANISMS

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Sometimes it is desirable to have a method for cultivating bacteria or fungi on inorganic gels which contain only added nutrient solutions of known composition. This may be the case, for instance, when one wants to investigate substances which are essential for, or produced by, the microorganisms. Also in some microbiological assay methods (for instance, determinations of vitamins and amino acids) such inorganic gels are useful. As is well known, the widely used agar-agar is an organic material, varying in composition and containing traces of substances which may have an influence on the growth of the microorganisms. A few microorganisms also liquefy agar-agar. Sometimes silicic acid jellies have been prepared of sodium silicate and hydrochloric acid, but the preparations are troublesome.

An earlier article described silica gels, for bacteriological purposes, which were easily prepared from *ortho*-silicic acid tetramethyl ester, $Si(OCH_3)_4$ (Ingelman and Jullander: Nature, 156, 272). Unfortunately this methyl ester is not obtainable in many countries. Therefore, we have now prepared silica gels for the same purpose from *ortho*-silicic acid tetraethyl ester, $Si(OC_2H_5)_4$, which is a common industrial product in, for instance, the United States. This silico compound gives, with water, silicic acid and ethanol. As, however, the ethyl ester is not so easily soluble in water as the methyl ester, the method has to be altered to obtain a firm, coherent, clear gel. We propose the following method:

One volume of $Si(OC_2H_5)_4$ is mixed with 1 volume of alcohol (ethanol). Into this solution is poured 6 volumes of water. The water is added in portions, with thorough mixing. Of course, the proportions can be altered a little to obtain jellies with varying rigidity. As a precaution against air bubbles in the jellies, "boiled out" water may be used. When the water is poured into the solution, there appears a slight turbidity. The mixture is therefore centrifuged until it becomes clear and then poured into tubes, petri dishes, or other suitable vessels. The solution is heated 30 to 40 minutes at 120 C in the autoclave. The solution then becomes a gel, clear as glass. After the heating, the gels must not be cooled too fast because the gel is then apt to crack. Water is poured over the gel and sometimes replaced with new water, so that the remaining ethanol diffuses away from the gel into the water and thus is removed. Then the water is replaced with a suitable nutrient solution and the tubes are left some time so that the nutrient substances diffuse into the gel. If one does not work under sterile conditions, the gels must be kept at a temperature high or low enough to prevent the growth of microorganisms. If the tubes are not sterile, they are then autoclaved in the usual way. The jellies are now ready to be used.

The silicic acid jellies are more apt to dry up than is agar-agar; hence they should be kept in moist air, for example, in a closed jar with some water in the bottom.

In order to test the silica jellies obtained in this way, we cultivated several different microorganisms on them with good results.

The authors thank Professors T. Svedberg and A. Tiselius for the privilege of being allowed to conduct the experiments in their laboratories. The silico ester used here was from the Uddeholm Company (Skoghallsverken), Sweden.

ADVANTAGES OF INCUBATION AT 30 C FOR THE STUDY OF STAPHYLOCOCCI[1]

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Although staphylococcus medium no. 110 (Chapman, J. Bact., 51, 409) has given excellent results in this laboratory and in many other laboratories, it still has two disadvantages, viz., (1) the development of pigment is not optimum, because some cultures either become pigmented or become darker when they stand a few days on the laboratory table, and (2) flooding the plates with ammonium sulfate solution for the Stone reaction is undesirable in several respects. A solution of the latter problem will be reported elsewhere. The former appears to have been solved by incubating the cultures at 30 C instead of the conventional 37 C.

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[1] Aided by grants from the Ophthalmological Foundation, Inc.

A series of cultures tested under these conditions showed deeper pigment in many instances, decidedly improved coagulation of blood, and no interference with the Stone reaction nor with acid production from mannitol, both of which were about as intense as at 37 C.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

TEXAS BRANCH

DALLAS, TEXAS, NOVEMBER 16 AND 17, 1946

POLIOMYELITIS (LANSING) CONTACT INFECTION IN MICE. *Christine Zarafonetis, S. Edward Sulkin, and Cleo Hausman Terry*, Department of Bacteriology and Immunology and Virus Research Laboratory, Southwestern Medical College, Dallas.

During 63 days of observation, only 1 of 50 white Swiss mice in contact with a similar number of animals inoculated intracerebrally with the virus of mouse-adapted Lansing poliomyelitis developed a paralytic infection. Virus was recovered from this animal but not from 3 other contact mice which died. The appearance of diarrhea among some of the animals in the cage shortly before the paralytic infection occurred suggested the importance of nonspecific factors of resistance; these possibilities are under further investigation. Subsequent challenge inoculations of virus revealed cerebral resistance among contact mice significantly more marked than that among controls of similar age and weight, but somewhat less marked than that among mice surviving previous intracerebral injection of the same virus. The virus recovered from the paralyzed animal, and subsequently inoculated into other mice, differed from the virus of spontaneous mouse encephalomyelitis on the basis of incubation period, site and extent of paralysis, and mortality rate among paralyzed animals. Immunologic studies to identify the agent as mouse-adapted Lansing poliomyelitis virus are still in progress.

FURTHER OBSERVATIONS ON THE INTESTINAL BACTERIA OF GUINEA PIGS FED WITH PARA-AMINOBENZOIC ACID. *Dorothy M. Whitney*. Department of Preventive Medicine and Public Health, The University of Texas School of Medicine, Galveston.

The investigation of the intestinal flora of guinea pigs before and after feeding *p*-aminobenzoic acid is the subject of this study.

Animal feces were suspended in 1:10 dilutions, and serial tenfold dilutions were inoculated into various media. In "normal" animals, the gram-positive bacteria predominated. In this group, bacilli, streptococci, and spore-bearing anaerobes occurred in the order named. Of the gram-negative bacilli, *Aerobacter aerogenes* and *Escherichia coli* dominated. In addition, non-lactose-fermenters such as *Alcaligenes*, *Pseudomonas*, and *Proteus* were found.

Guinea pigs treated with 2 per cent PABA in the diet for 48 hours showed a marked decrease in the number of gram-positive and gram-negative bacilli. In certain cases no reduction was noted, but a definite suppressive and apparently selective action was observed. The 48-hour treatment with PABA resulted in an almost complete inhibition of all gram-negative bacilli with the exception of *E. coli*.

A prolonged feeding with PABA (6, 8, and 10 days) resulted in a return to the normal flora. A small dosage of PABA (0.1 per cent) over 6 days resulted in increased streptococci counts.

PERMEABILITY OF THE LACTATING BOVINE MAMMARY GLAND TO SULFONAMIDES. *V. T. Schuhardt, T. B. Carroll, L. J. Rode, and Helen Lacy*, The Brucellosis Research Project of The Clayton Foundation and The University of Texas.

In preliminary and repeat experiments, sulfanilamide, sulfathiazole, sulfadiazine, sulfamerazine, sulfapyrazine, 2-sulfanilamido-5-bromo pyrimidine, and 2-sulfanilamido-5-chloro pyrimidine each were administered orally in capsules to 2 cows. Sulfapyridine was administered to 4 cows. Each cow received 90 grams of the appropriate drug in 3 equal doses at intervals of 4 hours. Blood and milk samples, collected from each cow at 6, 10, 22, 34, and 46 hours after the initial drug dose, were tested for free sulfonamide concentrations, and the last three samples of both blood and milk were tested for total sulfonamide concentrations.

Sulfathiazole, sulfapyrazine, and sulfadiazine attained maximum blood levels of less than 5 mg per cent of the free drug, and only the latter was demonstrable in the milk, attaining a concentration of 1.1 mg per cent. All the other drugs attained maximum blood levels ranging from 5 to 13 mg per cent free drug. Only sulfanilamide and sulfapyridine, however, came through the mammary gland in concentrations approaching those found in the blood, and sulfapyridine reached higher levels in both blood and milk.

FURTHER STUDIES ON THE EFFECT OF STERILIZING GLUCOSE IN CULTURE MEDIA ON GROWTH OF MICROORGANISMS: UTILIZATION OF CYSTINE BY LACTIC ACID BACTERIA. *C. E. Lankford, Cathryn Swausch, and Joanne Macow Ravel*, University of Texas School of Medicine, Galveston.

Previous studies have shown that autoclaving glucose or other reducing sugars in certain culture media for the gonococcus renders the peptone cyst(e)ine partially unavailable for growth. With the H_2O_2-treated peptone media of Lyman *et al.* (Arch. Biochem., **10**, 427), the growths of *Lactobacillus arabinosus*, *Lactobacillus casei*, and *Leuconostoc mesenteroides* were compared at different dosage levels of cystine with glucose autoclaved separately and in the medium. When glucose was autoclaved in the medium at pH 6.8 for 15 minutes at 15 pounds, there was a decrease in response to cystine ranging from 50 to 95 per cent. Increasing pH or autoclaving time markedly increased the extent of inactivation of cystine. With glucose autoclaved separately, smooth and reproducible curves were obtained, particularly with *L. mesenteroides*, which produces one-half maximum response at 0.3 μg per ml. Lower maxima with glucose sterilized in the medium suggests partial "inactivation" of other essential nutrilite(s), possibly as a result of combination of the reactive functional groups with aldehyde degradation products of glucose.

PRELIMINARY STUDIES ON THE POTENTIAL PATHOGENICITY OF BACILLUS CEREUS. *Kenneth L. Burdon*, Department of Bacteriology and Immunology, Baylor University College of Medicine, Houston.

PENICILLIN PRODUCTION BY A THERMOPHILIC FUNGUS. *L. J. Rode, Jackson W. Foster, Helen Lacy, and V. T. Schuhardt*, Department of Bacteriology and The Brucellosis Research Project of The Clayton Foundation, The University of Texas, Austin.

THE EFFECT OF BISMUTH ON THE ANTIBIOTIC ACTIVITY OF ASPERGILLIC ACID. *Andres Goth*, Department of Physiology and Pharmacology, Southwestern Medical College, Dallas.

ERADICATION OR SUPPRESSION OF BORRELIA RECURRENTIS IN THE VECTOR O. TURICATA BY PENICILLIN. *W. M. Fisher*, Department of Public Health and Preventive Medicine, Baylor University College of Medicine, Houston.

BIOCHEMISTRY OF ACTINOMYCETES. *C. Willard Lewis and Orville Wyss*, Department of Bacteriology, The University of Texas, Austin.

PROTEOLYTIC ENZYMES OF BACTERIA. *Dorothy L. Wallace, J. R. Stockton, and Orville Wyss*, Department of Bacteriology, The University of Texas, Austin.

THE EFFECT OF VARIOUS ESSENTIAL OILS ON MICROORGANISMS. *A. Packchanian*, Laboratory of Microbiology, The University of Texas Medical School, Galveston.

RESULTS OF COMPLEMENT FIXATION TESTS FOR ENDEMIC TYPHUS ON RATS FROM DDT-DUSTED AREAS. *Billie Jo Colquitt, J. N. Murphy, Jr., and J. V. Irons*, Bureau of Laboratories, Texas State Department of Health, Austin.

MICHIGAN BRANCH

East Lansing, Michigan, November 20, 1946

Newcastle Disease. *Charles H. Cunningham.*

Accurate differential diagnosis requires laboratory procedures for the isolation of the virus, serum neutralization tests, and hemagglutination and hemagglutination inhibition tests. Embryonated chicken eggs provide an excellent cultural medium. Early clinical cases are the best for the recovery of the virus. Brain and spleen are the organs of choice, although other organs, body discharges, and egg yolk may be used. With the onset of nervous symptoms the virus is usually no longer recoverable.

The virus retains its infectivity under the usual methods of storage. Inactivation by formalin, ultraviolet light, and heat does not materially alter its hemagglutination activity.

Several types of vaccines elicit immunity response, but the protection is of short duration. Vaccinated birds may resist challenge exposure from 2 to 4 days following vaccination, although demonstrable antibodies are not present until the sixth or eighth day. Refractivity to infection and immunity response are associated with growth and maturity.

Congenital passive immunity may be demonstrated in embryos and chicks from recovered or vaccinated hens. Immunity in such chicks closely parallels the period of yolk absorption.

Observations on Canine Leptospirosis in the Lansing Area. *J. P. Newman,* Department of Bacteriology and Public Health, Michigan State College.

During the past 15 months approximately 500 samples of canine blood have been examined for a present or past infection of leptospirosis. The following laboratory methods have been employed: darkfield examination, Giemsa stain preparations of whole blood and serum, macroscopic agglutination test, microscopic agglutination test, guinea pig inoculation, and culture using Verwoort's medium (Schüffner's modification).

It is evident in the work done thus far that, because of the individual limitations and pitfalls encountered with the foregoing laboratory diagnostic procedures, one must utilize two or more in an attempt to arrive at an accurate diagnosis of leptospira infection in the canine. The two procedures which will give the most accurate picture of present or past infection are the microscopic agglutination test and culture.

Equine, bovine, ovine, and porcine serums have been used in an attempt to find a more readily available serum to replace the rabbit serum employed in Verwoort's medium, with little if any success. Growth was very poor, if any, in the medium containing the foregoing serums.

Serological studies employing the microscopic agglutination test were made, indicating an approximate 29 per cent canine infection in the Lansing area, of which 27 per cent are *Leptospira canicola* infections and 2 per cent *Leptospira icterohemorrhagiae.*

Observations on the Variant Types of Corynebacterium diphtheriae: Antigenic Analysis. *Howard E. Lind,* Dow Chemical Company, Midland, Michigan.

Antigenic analysis, consisting of two phases of investigation—(1) agglutinin production against bacterial cultures, and (2) precipitin production against alkali-soluble protein extracts of the microorganisms—were made in an attempt to show some degree of inherent relationships or common identity when the other characteristics differed. It concerned the original smooth virulent parent colony, 4 nonvirulent parent type variants, and 4 nonvirulent small type variants.

Antigenically, the antiprotein and antibacterial serum of the parent type indicated similarity with the parent type variant but a distinct dissimilarity with the small variants. The parent type variants in demonstrating a low degree of cross reaction suggest alteration in the composition of the protein. This substance in the small variants is altered beyond detection or is destroyed completely, inasmuch as no cross reaction with the parent antiserum was observed.

Preliminary findings suggest that the quantity of protein in the alkali-soluble fraction of *Corynebacterium diphtheriae* is directly correlated with those strains that maintain virulence.

NORTHWEST BRANCH

STATE COLLEGE OF WASHINGTON, PULLMAN, WASHINGTON, NOVEMBER 23, 1946

THE REAL AND THE APPARENT BACTERICIDAL EFFICIENCIES OF THE QUATERNARY AMMONIUM COMPOUNDS. *Ernest C. McCulloch*, State College of Washington, Pullman, Washington.

Marked commercial interest now is being shown in the quaternary ammonium compounds, which are used for the disinfection of skin and mucous membranes, for the cold "sterilization" of minor surgical instruments, as bactericides for eating utensils and drinking glasses, and as general disinfectants.

Plate counts of bacterial suspensions exposed to quaternary ammonium compounds show a very rapid initial decrease in plate count numbers, followed by a much less rapid decrease. The velocity of

$$\text{disinfection, } K = \frac{1}{\text{time}^2 - \text{time}^1} \cdot \frac{\text{Log plate count at time}^1}{\text{Log plate count at time}^2}$$

has been observed as 0.43 during the first minute, as 0.37 during the second minute, and as 0.0004 between the second and twenty-fourth hours. In milk, the addition of a quaternary ammonium compound produces a very rapid decrease in plate count numbers, which after several days' incubation is followed by a marked increase which may exceed the original inoculum.

The hypothesis is advanced that the very rapid initial decrease in plate count numbers reflects the agglomeration of the exposed organisms and their adherence to the sides of the tube, as well as actual killing. When the FDA technique is used, the agglomerated organisms may not be picked up by the 1:100-ml loop; they may adhere to the loop and not remain in the subculture medium; and in the absence of particulate material in the subculture medium they may stay coated with the quaternary ammonium compound and remain in a condition of bacteriostasis.

A surface-active bacteriostat, which forms a persistent film and has low toxicity to tissues, may have definite value in clinical medicine. Also, in sanitizing certain types of food-handling equipment, such a bacteriostat might have definite, but limited, use. As bactericides, these compounds need to be reinvestigated.

CELLULOSE-DECOMPOSING BACTERIA FROM THE RUMEN OF CATTLE. *R. E. Hungate*, State College of Washington, Pullman.

THE BACTERIOLOGY OF SPRAY-DRIED WHOLE MILK POWDER. *J. F. Coal*, State College of Washington, Pullman.

THE OXYGEN REQUIREMENTS OF MOLDS. *N. S. Golding*, State College of Washington, Pullman.

STUDIES ON A SPECIES OF TRICHOSPORON. *C. H. Drake*, State College of Washington, Pullman.

STUDIES ON THE EPIDEMIOLOGY OF SPOTTED FEVER. *W. L. Jellison*, USPHS, Rocky Mountain Laboratory, Hamilton, Montana.

SOME ASPECTS OF THE POLIOMYELITIS PROBLEM. *C. A. Evans*, University of Washington, Seattle.

KENTUCKY-TENNESSEE BRANCH

University of Tennessee, Knoxville, Tennessee, November 16, 1946

Microbial Fouling of Zeolite Water Softeners. *W. L. Williams*, Louisville Water Company, Louisville, Kentucky.

A water treatment plant treating a highly contaminated river water with lime and ferric sulfate for carbonate hardness reduction, followed by zeolite softening, used insufficient chlorination on the initial runs, thereby seeding the softener beds with *Aerobacter aerogenes*. By the application of break-point chlorination to the raw water intake, sterile conditions were thereafter maintained throughout the treatment until the water reached the softeners. The carbonaceous zeolite in the softeners quickly absorbed the chlorine residual, and bacterial growth flourished throughout the softeners, finally causing the beds to jell and channel on regeneration, with the result that the softeners failed to function. Water leaving the filters appeared to be clear, but the Sedgwick-Rafter concentration of samples, followed by microscopic examination, showed that dead organic matter was passing through the filters, thus constantly supplying nutrient material for the growth of bacteria. Bacterial counts on water leaving the softeners were extremely high, *Aerobacter aerogenes* being the predominating organism. It was always present when short softener runs were experienced. Several methods of backwash and two sterilizing compounds were effective only temporarily in removing contamination from softener beds.

The Use of Sodium Azide for Determining the Fermentative Ability of Yeast Developed Under Different Oxygen Tensions. *M. C. Brockmann*, Joseph E. Seagram and Sons, Inc., Louisville, Kentucky, *and T. J. B. Stier*, Department of Physiology, Indiana University, Bloomington.

If the rate of fermentation by yeast is limited primarily by overaccumulation of high energy phosphate bonds, conventional procedures are inadequate for evaluating fermentative ability. In the presence of 0.002 M azide there is a marked elevation in the rate of glucose utilization per unit cell population. This elevated rate remains constant for relatively long periods, presumably because azide interferes with the generation of high energy phosphate bonds and thereby releases the fermentative processes from the consequences of overaccumulation of these bonds.

On the basis of rates of glucose utilization in CO_2-sparged glucose yeast extract KH_2PO_4 medium containing azide (0.002 M), yeast cells developed under aeration have about one-half the fermentative activity of cells developed in CO_2-sparged medium. Cells grown in cotton-plugged flasks develop 70 to 80 per cent of the activity of cells grown under CO_2 sparging.

For yeast developed under different oxygen tensions, the rate of glucose utilization per unit cell population appears to be a more adequate expression of activity than a corresponding rate based on unit cell weight.

The Complement Fixation Test for Lymphogranuloma as a Diagnostic Procedure. *Anna Dean Dulaney and Henry Packer*.

The complement fixation test for lymphogranuloma venereum was conducted on 640 sera from diversified sources. Positive reactions in serum dilutions of 1:5 were obtained with 8.7 per cent of 148 Wassermann-negative individuals with no evidence of venereal infection, 2 per cent of 42 children with febrile disease, none of 22 children with congenital syphilis, 57 per cent of 81 patients with neurosyphilis, and 70 per cent of 214 patients with anogenital lesions of various types.

The nonspecific reactions in the nonvenereal group were correlated in most instances with upper respiratory infections.

Titration studies showed that positive reactions in the nonvenereal groups were characterized by low titers (1:5 to 1:20) whereas 56 per cent of the sera from the venereal groups yielded titers of 1:40 and above.

It is concluded that the complement

fixation test for lymphogranuloma venereum is of diagnostic value if sera are tested routinely in dilutions of 1:40. Such titers are in general diagnostic of lymphogranuloma venereum and tend to screen out nonspecific reactions due to early syphilis or other infectious diseases.

PHASE VARIATION IN PARACOLON ORGANISMS. *Mary G. West*, Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington, Kentucky.

The phase variation of Andrewes was found in a group of lactose-fermenting organisms bearing slight serological relationship to the *Salmonella* genus.

In some instances one phase of these paracolons could be well represented by *Salmonella* antigenic factors. In other instances the phases were found to bear only slight antigenic relationships to *Salmonella* organisms.

The discovery of phase variation among a group of organisms outside the *Salmonella* genus is significant in that this phenomenon is a factor which should be considered in the serological classification of paracolon and coliform organisms.

A STUDY OF VARIABILITY IN DUPLICATE STANDARD PLATE COUNTS AS APPLIED TO MILK. *J. L. Courtney*, Oak Ridge Department of Health, Oak Ridge, Tennessee.

A total of 299 duplicate counts were made on raw milk. Of these, 278 varied less than 50 per cent; 18 between 50 and 100 per cent; 3 over 100 per cent, the highest being 191 per cent. The average variation was less than 20 per cent. It seems that many of the extreme variations in standard agar plate counts are a result of failure to appreciate the importance of care in every detail. This conclusion seems more obvious when we recall statements often made that there is no point in being careful with certain phases of the technique, or that there is no point in doing something a certain way because the error inherent in the method is greater than the error which will be introduced.

Our limited study indicates that the human error may introduce extreme variations, and yet the result is seemingly accepted as the normal variation of the method. In the light of the abuse so often heaped upon this procedure, we feel that an improvement in the technique will be rewarded by more accurate results.

PROPOSED CHANGES IN INCUBATION TEMPERATURES FOR STANDARD AGAR PLATE COUNTS. *James D. Brew*, University of Tennessee.

The temperature of incubation for estimating bacteria by the agar plate method has long been a controversial issue. Forty years ago incubation was 5 days at 20 C, and 2 additional days at 37 C. The theory was that since these temperatures represented the optimum for most bacteria, including the pathogens, more accurate results would be obtained. A 7-day incubation period, however, proved to be impractical. The incubator space in most laboratories was limited; another objection was the long wait for results. Incubation at 37 C for 48 hours was finally agreed upon as being most satisfactory.

Recently, workers in milk control laboratories observed greater variabilities in estimates made at 37 C than at lower temperatures. Apparently, 37 C is close to or possibly above the maximum growth range for some bacteria; also the temperatures in different incubators vary more widely in the range of 37 C than at lower temperatures. Not only do temperatures of incubators of different manufacturers vary, but there may be variations at different points inside any incubator. Some 37 C incubators may run as high as 45 C, which is sufficiently high to inhibit the growth or possibly kill some organisms. The proposed change is to lower the incubation temperature to 32 C for 48 hours. Pederson and others found the variability in estimates at 32 C to be about 4 per cent, whereas at 37 C it was about 25 per cent. Furthermore, the total number estimated at 37 C averages about 50 per cent lower than that obtained at 32 C.

THE FUNGISTATIC EFFECTS OF THE FATTY ACIDS ON SPECIES OF TRICHOPHYTON. *Emanuel Grunberg*.

The fungistatic action of the series of fatty acids (formic acid to undecylenic acid) at various pH levels was investigated. It was determined that at pH 5.5 and pH 6.5 the potency of each acid increased with the addition of each methyl group according to Traube's rule. At pH 7.5 the potency of the acids from formic acid to valeric acid increased also according to Traube's rule; however, between valeric acid and caproic acid, there seems to be a break, with caproic and the subsequent acids much more potent than would be expected if potency increased according to this same constant. The potency of the acids at pH 8.5 is essentially the same as it is at pH 7.5.

Four strains of *Trichophyton gypseum* and four strains of *Trichophyton purpureum* were employed. Although it has been claimed that there is a difference in resistance to fungistatic agents between the two species, none was apparent in *in vitro* tests with the fatty acids.

A consideration of the results seems to indicate that the fungistatic activity of the fatty acids can be correlated with the undissociated fraction.

A CAPSULE-DISSOLVING FACTOR. *James C. Humphries*, Department of Bacteriology, University of Kentucky, Lexington, Kentucky.

Phage lysates of *Klebsiella pneumoniae*, type A cultures, freed from phage by ultrafiltration, contain a factor which removes the capsule from type A cells. This factor is specific, failing to remove the capsule from the types B and C Friedlander and *Aerobacter aerogenes* strains tested.

Capsule removal was demonstrated as follows: (1) Ultrafiltrates applied to the surface of agar plate culture produce greatly reduced opacity in the growth film. (2) Ultrafiltrate-treated growing cells or formalin-killed cells are no longer agglutinable by type-specific "M" sera, are agglutinable by "S" phase sera, and are greatly reduced in cell volume (Hopkins tube technique). (3) Ultrafiltrate-treated growing cells are rendered susceptible to lysis by an "S" culture phase bacteriophage.

In these crude preparations the principle is (1) stable near neutrality but rapidly inactivated in weak acid solutions; (2) destroyed by 85 C for 10 minutes, but resistant to 75 C for 30 minutes; (3) nondialyzable through cellophane, and not precipitated by the dialyzing process; and (4) precipitated following 50 per cent ammonium sulfate saturation.

SOME OBSERVATIONS DEALING WITH CORRELATIONS OF RESULTS OF STANDARD KAHN TEST AND THE QUANTITATIVE KAHN TEST. *Cooper Brougher, George M. Cameron, Rufus Buchanan, and Kent Roark*, State Department of Health, Nashville, Tennessee.

THERMAL SHOCK IN THE ISOLATION OF STREPTOMYCES. *A. L. Pollard*, University of Tennessee, Knoxville, Tennessee.

THE OCCURRENCE OF SALMONELLA ANTIGENS IN A COLIFORM BACTERIUM. *Alice B. Moran*, Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington, Kentucky.

EASTERN PENNSYLVANIA CHAPTER

ONE HUNDRED AND NINETIETH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, PHILADELPHIA, PENNSYLVANIA, NOVEMBER 26, 1946

THE EFFECT OF ULTRAVIOLET IRRADIATION ON VARIOUS PROPERTIES OF INFLUENZA VIRUSES. *Werner Henle and Gertrude Henle*, Children's Hospital, Philadelphia, Pennsylvania.

The effect of ultraviolet irradiation on various properties of the influenza viruses types A and B has been analyzed. The studies involved propagation and interference in the allantoic sac of the chick embryo, inhibition of embryonic development, toxicity for white mice, hemagglutination including the adsorption-elution mechanism, immunizing capacity for mice, and, finally, complement fixation activities in the presence of antibodies to the 600S antigen (human convalescent and post-vaccination sera) and the 30S antigen (convalescent sera only). It could be shown that the various activities of the influenza viruses were affected by irradiation at different rates, indicating that they are based, at least in part, on different constituents of the virus particle. On account of these differences in the susceptibility of the various properties to ultraviolet light it was possible (a) to differentiate between the interference phenomenon as observed in the allantoic sac and the development of nonagglutinability in red cells by either homologous or heterologous fresh virus, and (b) to separate individual steps involved in the mechanism of infection of susceptible host cells. The implications of these findings were discussed.

Familial Nonspecific Serologic Reaction for Syphilis. *Arthur G. Singer, Jr., and Fred Boerner*, Graduate Hospital and Lankenau Hospital, Philadelphia, Pennsylvania.

The existence of nonspecific positive reactions to a serologic test for syphilis is now generally accepted. The majority of false positive serologic reactions are traceable to an infectious disease, an injection, or treatment of some sort. However, the case in question deals with a family, several members of which show persistent false positive tests that cannot be attributed to any of the usual causes.

The routine prenatal serologic tests for syphilis on a 35-year-old white female of Italian birth and four to five months pregnant repeatedly showed a positive Kline test but a negative complement fixation. The husband was found to give entirely negative serologic results; the serum of the three oldest children, however, gave reactions identical with that of the mother, i.e., positive Kline, negative complement fixation. Neonatal serologic studies on the youngest child were entirely negative. The entire family were Rh-positive, but the father and youngest child were found to be in blood type group O, whereas the mother and three oldest children belonged to group A.

Quantitative serological tests were conducted on the mother and three oldest children; positive results were obtained in the flocculation test, but the complement fixation test gave negative results. In each case, the results fell into class 5, group 2, a doubtful classification according to Boerner's system of classification. The father and youngest child remained negative in both tests, falling into class 1, group 1, negative.

These studies, conducted over a period of two years, suggest the existence of a possible mechanism of hereditary transmission of the factor responsible for the nonspecific positive reaction.

Inhibition of Division in the Protozoan Tetrahymena by Antisera and Bacteria. *Elizabeth H. Fowler and James A. Harrison*, Department of Biology, Temple University, Philadelphia, Pennsylvania.

Further study has been made of the interesting serologic reaction with the free-living ciliate, *Tetrahymena*, reported by the authors in 1945. The antibody which interferes with division without interrupting other growth processes and leads to the abnormal formation of pairs and chains of cells, as well as monsters, may be removed by absorption. An unsuccessful attempt was made to produce a similar reaction in these organisms by exposure to 195 strains of coliform bacilli by the method of Chatton and Chatton reported in 1925.

EASTERN NEW YORK BRANCH

Albany, New York, December 6, 1946

The Relation Between Induced Resistance to Penicillin and Oxygen Utilization. *W. D. Bellamy and J. W. Klimek*, Sterling-Winthrop Research Institute, Rensselaer, New York.

In this laboratory the resistance to penicillin of *Staphylococcus aureus* 209P has been increased over 60,000 times by 64 serial transfers in broth containing penicillin, the amounts of which were increased from 0.00006 mg per ml to 4 mg per ml (Klimek *et al.*: J. Bact., 51, 550). We have found that this resistant strain has lost the ability to grow anaerobically. The rate of aerobic growth is from one-half to two-thirds of that of the penicillin-sensitive parent strain.

Under similar conditions, three other organisms, which normally obtain energy for growth by anaerobic processes, e.g., *Streptococcus faecalis* 10Cl, *Streptococcus agalactiae* 68C, and *Clostridium perfringens* M, were found to develop little resistance to penicillin. The resistance of *S. faecalis* increased 11 times in 47 transfers, that of *S. agalactiae* 6 times in 24 transfers, and that of *C. perfringens* M 10 times in 25 transfers.

It appears that penicillin interferes with an essential step or steps in anaerobic metabolism. Those organisms which were unable to develop or utilize an alternative energy mechanism did not develop resistance to penicillin.

INACTIVATION OF THE GERMICIDAL ACTION OF QUATERNARY AMMONIUM COMPOUNDS. *C. A. Lawrence*, Winthrop Chemical Company, Inc., Rensselaer, New York.

To be considered a suitable inactivator for the antibacterial action of quaternary ammonium germicides the agent or process used must meet certain prerequisites: (1) the method should, as completely as possible, neutralize the bacteriostatic and bactericidal actions of the quaternaries; (2) it should maintain the state of inactivation for a prolonged period of time; and (3) the neutralizing agent itself should not possess antibacterial properties. Evidence is presented to show that several anionic detergents, including soaps, would not meet the requirements given. In addition, a series of compounds known to give a precipitate in the presence of quaternary ammonium solutions failed to inactivate completely the germicidal action of the latter. A sulfonic acid derivative, the sodium salt of symmetric bis(*meta*-amino-benzoyl-*meta*-amino - *para* - methylbenzoyl - 1 -naphthyl-amino - 4,6,8 - trisulfonic acid) carbamide, more closely meet the requirements for an inactivator for squaternary ammonium compounds.

ACCESSORY GROWTH SUBSTANCES INFLUENCING COLONY CHARACTERISTICS AND MACROCONIDIAL FORMATION OF MICROSPORUM AUDOUINI. *Elizabeth L. Hazen*, Branch Laboratory, Division of Laboratories and Research, New York City.

THE PROTECTIVE VALUE OF THE VOLE BACILLUS (WELLS) AS COMPARED WITH BCG AGAINST TUBERCULOUS INFECTION. *Konrad Birkhaug*, Division of Laboratories and Research, Albany.

CONNECTICUT VALLEY BRANCH

YALE UNIVERSITY, NEW HAVEN, CONNECTICUT, DECEMBER 7, 1946

GENETIC EVIDENCE FOR SEX IN BACTERIA. *Joshua Lederberg and E. L. Tatum*, Osborn Botanical Laboratory, Yale University, New Haven, Connecticut.

Following a discussion of a previous publication (Nature, **158**, 558) a demonstration was presented illustrating the recombination of genetic factors in *Escherichia coli*.

THERAPY OF TRYPANOSOMIASIS. *Benjamin A. Rubin*, Department of Bacteriology, Yale University School of Medicine, New Haven, Connecticut.

Prodigiosin and synthetic pyrrole derivatives were found to have selective trypanocidal activity *in vitro*, but not *in vivo*. No antibacterial effects were demonstrable.

Oxygen analogues in the form of natura and synthetic lactones were highly effective *in vitro* and in certain cases provided permanent cures for *Trypanosoma equiperdum* infections in mice. The level of this activity depended upon the location of unsaturated linkages and upon the nature of the substituents.

The locus of trypanocidal activity was in one case shown to be different from that of the antibacterial effect. The parent substance of "anemonine," the synthetic 2-pentene 1:4 olid, had equal trypanocidal activity but had none of the substantial antibacterial effect of anemonine.

The *in vitro* trypanocidal activity of some of the lactones (particularly "parasorbic" acid) could be reversed by *beta*-alanin and to a lesser extent by *alpha*-alanine, but not at all by calcium pantothenate or pantolactone.

Variation in Tissue Specificity of the Rous Chicken Sarcoma Virus Following Growth of the Tumor in the Mammalian Eye.

Edward W. Shrigley, Bacteriology Department, Yale Medical School, New Haven, Connecticut.

It has been shown that the Rous sarcoma will grow in the anterior chamber of the guinea pig eye. Following residence of 12 days in this host, the causative virus of this tumor has been found to be altered in its tissue specificity as evidenced, on subsequent inoculation into chicks, by the increase in frequency of periosteal tumors.

In the present study it has been possible to grow the Rous sarcoma in the anterior chamber of the mouse eye. Twenty-three of the 31 A strain mice inoculated intraocularly with the tumor tissue showed growth of the neoplasm, as manifest by increase in size and the presence of mitotic figures. All ocular growths remaining in mice up to 15 days produced typical sarcomas when reinoculated into chicks.

The incidence in chicks of bone lesions produced by the injection of the guinea-pig-passage strain of tumor compared with the frequency of similar lesions in birds inoculated with mouse-passage material showed that the virus varied while in the guinea pig environment but was not altered, according to our tests, during mouse passage. The explanation for these results must await further study.

The Isolation of Paracoccidioides brasiliensis from a Case of South American Blastomycosis.

Rosalie Ferguson and Margaret F. Upton, St. Luke's Hospital, New York City.

The fungus *Paracoccidioides brasiliensis* was isolated from a case of South American blastomycosis in a 23-year-old man who contracted the disease in Colombia about 3 months before coming to this country. At St. Luke's Hospital, New York City, the fungus, showing multiple budding, was observed in sections from an axillary lymph node. The mycelial phase was grown from a lymph gland upon blood veal agar in 4 weeks, incubated at room temperature. Transfers to Bordet-Gengou medium incubated at 37 C were successful in growing, in 7 days, the multiple-budding yeast characteristic of the tissue form. This is probably the first isolation of this fungus reported in this country.

A Quantitative Method for the Determination of the Fungistatic Activity of Antiseptic Powders.

Marion B. Sherwood, Wellcome Research Laboratory, Tuckahoe, New York.

NORTH CENTRAL BRANCH

UNIVERSITY OF MINNESOTA, MINNEAPOLIS, MINNESOTA, NOVEMBER 22 AND 23, 1946

Some Observations on the Free-living Leptospiras of Minnesota Waters.

B. H. Hoyer, University of Minnesota, Department of Bacteriology and Immunology, Minneapolis, Minnesota.

Attempts were made to isolate free-living leptospiras. Of the enrichment methods tested, Walker's medium was found to give the best results. Walker's medium is 0.3 per cent egg yolk in 0.3 per cent agar. Modifications of this medium consisting of added phosphate buffer, cystine, or tryptose (Difco) gave results no better than, or poorer than, the plain medium.

Positive enrichments for free-living leptospiras were obtained in 17 out of 19 enrichment attempts using Walker's medium and the straw enrichment of Kitaoka. All types of waters were included: algae-filled ponds, muddy waters, clear leafy-bottomed ponds, rivers, and even sulfur springs. Positive enrichments were obtained in equal number at both 23 C and 37 C

Pure cultures of the free-living leptospiras may be obtained by a preliminary Berkefeld filtration followed by growing the filtrate on Walker's medium. Leptospiras and very small rods and vibrios appear in this stage, and purification may be made by dilution extinction.

Strains of the leptospiras may be separated on a solid tryptose (Difco) agar medium after preliminary purification as described above.

ASSIMILATION OF ACETATE BY YEAST. *A. G. C. White, L. O. Krampitz, and C. H. Werkman*, Industrial Science Research Institute, Iowa State College, Ames, Iowa.

Large increases in the fat content of metabolizing yeast (*Saccharomyces cerevisiae*) in the presence of sodium acetate were shown to be due to the assimilation of the intact acetate molecule. By the use of acetic acid labeled in the carboxyl group with a heavy isotope of carbon (C^{13}), the carbon from the acetate was shown to be present in both the fat and carbohydrate fraction of the yeast cells.

In one experiment normal acetate was supplemented by $NaHC^{13}O_3$; in a second experiment isotopic acetate was supplemented by normal bicarbonate. No isotope was found in the yeast fat in the first experiment, a fact which indicates that the C^{13} was not incorporated into the fat of the yeast cells by CO_2 fixation but rather by utilization of the intact 2-carbon chain.

THE CONVERSION OF 2,3-BUTYLENE GLYCOL TO ACETYLMETHYLCARBINOL IN BACTERIAL FERMENTATIONS OF GLUCOSE. *David Paretsky and C. H. Werkman*, Bacteriology Section, Iowa Agricultural Experiment Station, Ames, Iowa.

The yields of acetylmethylcarbinol (AMC) in the fermentations of glucose by *Aerobacter aerogenes* may be increased at the expense of 2,3-butylene glycol (2,3-BG). Methylene blue added toward the end of aerated glucose fermentations increases the amounts of AMC produced.

Aeration of fermentations under pressure also increases the ratio of AMC to 2,3-BG. The highest conversions of 2,3-BG to AMC are obtained when methylene blue is added to the fermentation in conjunction with pressure aeration.

THE MICROBIOLOGY AND CHEMISTRY OF CANNED BACON. *J. A. Ulrich, H. M. Tsuchiya, and H. O. Halvorson*, The Hormel Institute, University of Minnesota, Austin, Minnesota.

Because "heavy cure" bacon leaves much to be desired in palatability, microbiological and chemical studies have been made of the changes that occur during each of the commercial steps involved in the processing of bacon sides from "green bellies" to the finished "mild cure" product, and on storage at 37.8 C.

The bacterial population decreases during the curing and smoking processes. On storage at 37.8 C (100 F) the total bacterial and the lipolytic bacterial counts of canned bacon samples increased and subsequently decreased. The free fatty acid values and the ammonia N: total N ratios increased, while the nitrite concentrations decreased. The pH and peroxide values remained constant during the period of storage. Vacuum pack samples kept better than nitrogen pack samples, and the latter better than the carbon dioxide pack samples. The usual commercial practice of derinding after the smoking process gave a better keeping product than did derinding before this step.

Samples of "heavy cure" canned bacon withstood storage very well at the storage temperature of 37.8 C.

THE EFFECT OF DOSAGE ON INTERFERENCE BETWEEN DISTEMPER VIRUSES. *Cyril S. Stulberg and Robert G. Green*, Department of Bacteriology and Immunology, University of Minnesota Medical School, Minneapolis, Minnesota.

Interference between animal, plant, and bacterial viruses in their respective hosts has been demonstrated by many workers. The authors have previously described an interference between distemper viruses (Proc. Soc. Exptl. Biol. Med., **61**, 117; Science **103**, 497). A highly fatal canine distemper in young foxes produced by intranasal inoculation could be blocked during the incubation period by the intramuscular injection of a nonvirulent ferret-passage virus.

Further studies have now shown that foxes can be uniformly infected with virulent distemper by intranasal inoculation of 50 mg of infected tissue, and that varying dosages of the modified virus will interfere with the virulent distemper infection. Sixty red fox pups were injected with 50 mg of virulent distemper virus intranasally.

Seven days later 50 of the animals were divided into groups of 10, which were injected intramuscularly with modified distemper virus in dosages of 200, 100, 50, 25, and 10 mg, respectively. Ten animals kept as controls died of virulent distemper. The 50 foxes in the groups inoculated with modified virus exhibited definite symptoms of distemper but subsequently recovered, except that 1 fox died in the group inoculated with 200 mg of modified virus, 1 in the group inoculated with 100 mg, 4 in the group inoculated with 50 mg, and 2 in the group inoculated with 10 mg.

It appears that as little as 10 mg of modified distemper virus injected intramuscularly 1 week after intranasal instillation of virulent distemper virus will interfere with the course of an infection caused by the virulent virus.

EFFECT OF SPECIFIC ANTIBODY ON TRANSPLANTED MOUSE MAMMARY CANCER. *David T. Imagawa and Robert G. Green*, Department of Bacteriology and Immunology, University of Minnesota.

Green, Moosey, and Bittner (Cancer Research, 5, 538) have shown that the inciting agent of mouse mammary cancer is highly antigenic and, when inoculated into rabbits, produces specific antibodies which inactivate the agent. It has been possible in our laboratory to concentrate these antibodies from 4 to 10 times their original concentration by precipitation of the globulin fraction with 1.39 molar ammonium sulfate.

This concentrated antiserum has been found to have a definite inhibitory effect on the growth of transplanted mouse mammary cancer. Clumps of mouse mammary cancer cells were inoculated subcutaneously into the abdomens of mice. At the beginning of each experiment the tumor-bearing animals were divided into groups so that the average tumor size of each group was approximately the same. Treatment with antiserum was started after the tumors had been established and had attained the size of a small pea. Different routes of injection were used at varying time intervals. At the beginning and at various intervals of the experimental periods, the surface areas of the tumors of both control and treated animals were accurately calculated and compared.

It appeared that the growth of transplanted mouse mammary cancers was partially inhibited by the injection of concentrated mouse cancer antiserum, but the injection of normal rabbit serum and concentrated normal rabbit serum showed no noticeable effect. Injection of the specific antiserum into the tail veins of mice was the most effective method of injection.

SYNERGISM BETWEEN SOME ANIONIC WETTING AGENTS AND AZOCHLORAMID. *B. H. Hoyer and E. J. Ordal*, University of Washington, Department of Microbiology, Seattle, Washington.

In view of the synergistic action of some anionic wetting agents on the germicidal action of undissociated phenols, an attempt was made to demonstrate similar action using an oxidizing disinfectant. Because of its stability, azochloramid (N,N′-dichlorazodicarbonamidine) was used as the oxidizing type disinfectant. Three anionic wetting agents were used: sodium lauryl sulfonate, sodium tetradecyl sulfate, and the dioctyl ester of sodium sulfosuccinate. *Escherichia coli* and *Staphylococcus aureus*, used as the test organisms, were grown in shaken flasks and washed. Centrifuged cells were used in all experiments. Attempts to demonstrate synergism were made by using decrease in respiration, as determined by the Warburg respirometer, and by bacterial death, as determined by quantitative plate counts.

No evidence of synergism was found in the respiration studies using oxidation of lactate as the indicator. Quantitative counts gave definite evidence of synergism. The extent of synergism did not follow a definite trend, as in the case of the phenols, suggesting some mechanism in operation other than the action on undissociated hypochlorous acid resulting from the hydrolysis of azochloramid. Synergism apparently occurred to the greatest extent when both of the agents independently exerted some amount of germicidal activity.

ON THE MODE OF ACTION OF PENICILLIN. *L. O. Krampitz, M. N. Green, and C. H. Werkman*, Industrial Science Research

Institute, Iowa State College, Ames, Iowa.

An inhibitory effect of penicillin on the oxidation of neucleotides or nucleic acids by *Staphylococcus aureus* has been demonstrated. Penicillin has no effect on glucose oxidation or on the utilization of the constituents of a synthetic medium on which good growth can be obtained.

The reaction inhibited by penicillin has an RQ of 1.0. The measurable end products are CO_2 and acetic acid in a ratio of 3 to 1. Since 3 moles of oxygen are required for the oxidation, the dissimilation of a pentose, according to the following equation, is postulated.

$$C_5H_{10}O_5 + 3\ O_2 \cdot \rightarrow CH_3COOH + 3\ CO_2 + 3\ H_2O$$

Sodium ribonucleinate on oxidation by *S. aureus* shows the same ratio of end products. This reaction is inhibited by penicillin. Streptomycin has also been shown to exhibit an inhibitory action on the same reaction.

Yeasts in Decomposing Fleshy Fungi. *K. W. Anderson and C. E. Skinner*, Department of Bacteriology and Immunology, University of Minnesota.

Melibiose Broth from Raffinose by Fermentation in Yeast Taxonomy. *R. Bouthilet and C. E. Skinner*, University of Minnesota.

Inactivation of Neurotropic Viruses by Mechanical Agitation. *W. F. McLimans*, Naval Medical Research Institute, Bethesda, Maryland.

Experimental Therapy of Scrub Typhus with Methyl Thiamine Chloride. *W. F. McLimans and C. W. Grant*, Naval Medical Research Institute, Bethesda, Maryland, *and W. P. Larson*, University of Minnesota.

Comparison of Growth of Lactobacillus casei in Yeast Extract Media and Synthetic Media of Varying Composition. *M. R. Muedeking and H. O. Halvorson*, University of Minnesota.

Intramural Dissemination of Mold Spores. *C. M. Christensen*, Plant Pathology Department, University of Minnesota, St. Paul, Minnesota.

ILLINOIS BRANCH

Chicago, Illinois, January 17, 1947

Variation in Molds—Natural and Induced. *Kenneth B. Raper*, Fermentation Division, Northern Regional Research Laboratory, Peoria, Illinois.

Saprophytic molds are regularly characterized by marked natural variation, and in the genera *Aspergillus* and *Penicillium* this occurs at all levels of classification. Groups are established as a matter of convenience, but are seldom sharply delimited. Within these groups, species descriptions are centered upon fairly tangible differences, but intermediate and transitional forms are the rule rather than the exception. The more common species in turn are extremely variable, and individual strains or isolates often differ markedly in cultural and morphological characteristics. Cultures derived from single spores, long considered by mycologists as yielding the ultimate in strain individuality, afford little assurance of sustained stability. Such natural variation offers unusual opportunities for the experimental microbiologist, since species and strains vary not only in cultural and morphological characteristics, but in nutritional requirements, biochemical reactions, and in fermentative capacities as well. It also poses some problems, since most variations are in the direction of reduced activity, or, in fermentations, reduced yields. By careful selection, substrains characterized by some increased activity in desired directions can often be developed. This is generally limited, however, and it is necessary to employ external stimuli such as ultraviolet or X-ray radiation to secure further improvements. Variation in colony appearance and structure may or may not be correlated with changes in physiological activity.

SOME FACTORS AFFECTING THE STABILITY OF TYPE A (PR8) INFLUENZA VIRUS.

George F Forster, Venus Love, and Esther Carson.

The viability of this virus has been studied under certain conditions of laboratory maintenance, particularly (1) in storage at −60 to −70 C as a concentrate of allantoic virus, (2) in storage at 5 to 8 C as a lyophilized concentrate of allantoic virus, and (3) in storage at 5 to 8 C as allantoic virus diluted decimally in nutrient broth. Longevity was measured in terms of months under each of these methods of treatment. The chick red cell method of concentration was employed, and the character of the elution fluid was an important factor in the stability of the concentrates whether stored as such at −60 to −70 C, or lyophilized and stored at ordinary refrigerator temperatures. Elution into a 50:50 mixture of normal rabbit serum and nutrient broth or into inactivated allantoic fluid resulted in considerably greater stability than when elution was made into physiological saline or buffered (pH 7.4) saline. When unconcentrated allantoic virus was diluted in nutrient broth (10^{-1} to 10^{-7}) and these dilutions were stored at 5 to 8 C, deterioration was gradual over a period of many weeks. Mouse-killing potency was the criterion of viability.

A STUDY OF THE BACTERIAL FLORA OF THE NORMAL AND PATHOLOGICAL VAGINA AND UTERUS.

K. Eileen Hite, H. Close Hesseltine, and Louis Goldstein, Department of Bacteriology and Parasitology and the Department of Obstetrics and Gynecology, The University of Chicago and The Chicago Lying-In Hospital.

A study has been made of the aerobic and anaerobic bacterial flora of 248 patients of the Chicago Lying-In Hospital. The study included vaginal cultures from normal prenatal patients and patients having vaginal infections (trichomoniasis, moniliasis, and vaginitis of unknown etiology), and intra-uterine cultures from normal and febrile *post-partum* patients and from a few postabortal puerperae. In general, the flora of the vagina was similar in normal prenatal patients and in those with mycotic and nonspecific vaginitis. Aciduric rods were the predominant organisms. A variety of bacteria were isolated from the vagina of trichomoniasis patients and the uterine cavity of normal and febrile puerperae. The bacterial flora in the latter groups was similar.

AUTOLYZED BRAIN TISSUE AS A MEANS OF FACILITATING TRANSMISSION OF POLIOMYELITIS TO MICE.

Albert Miller, Chester L. Byrd, and Sidney O. Levinson, Serum Center, Michael Reese Research Foundation, Chicago, Illinois.

Most attempts to infect various animals with poliomyelitis monkey-passage strains or infected human tissues failed until Armstrong was able to adapt the Lansing strain from monkeys to cotton rats and from the latter to white mice. Since then many unsuccessful attempts to establish other monkey-passage strains in various rodents have been made using a variety of techniques such as rapid passage, brain trauma, hyperpyrexia, chilling, spreading factor, and use of immature animals.

Autolyzed brain tissue diluent prepared from normal CFW Swiss mice was found to shorten the incubation period and facilitate the transfer of poliomyelitis virus to CFW mice, hamsters, and rhesus monkeys. By means of this technique the Leon monkey-passage strain of poliomyelitis virus was successfully adapted to CFW mice. Proof of adaptation was shown by successful transfer to monkeys and neutralization in high dilution with human immune serum globulin. More recently several strains of poliomyelitis virus have been isolated in CFW mice from infected human stools and a spinal cord using autolyzed brain diluent.

COMPARATIVE BACTERIOSTATIC ASSAYS WITH ROSANILINE AND ITS PHENOLIC ANALOG (ROSOLIC ACID)

E. FISCHER AND R. MUÑOZ

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Certain relations between the chemical structure and the bacteriostatic action of di- and triphenylmethane dyes have been studied by Browning and Gilmour (1913) and by Kligler (1918). The findings of these workers are in agreement, as are likewise those of Thornberry (1931). Partially contrasting findings have been published by Fairbrother and Renshaw (1922), who used, however, a different technique, based on bactericidal effects. Their results will therefore not be considered here.

The experimental data of the above-mentioned authors, confirmed and somewhat extended by one of the writers and coworkers (Fischer, Garcés, and López, 1946, and unpublished experiments) permit the following conclusions to be deduced:

(1) The presence of three phenylic rings attached to the central methyl radical (i.e., the triphenylmethane structure) results in a considerably stronger action than does the presence of only two such rings (diphenylmethanes) (Kligler, 1918; Fischer *et al.*, 1946).

(2) The presence of an amino group attached to the third phenylic ring is of little or no significance, since diamino derivatives are nearly or completely as active as the corresponding triamino derivatives malachite green vs. methyl violet (Browning and Gilmour, 1913; Kligler, 1918; Thornberry, 1931).

(3) The methylating, and especially the ethylating, of the amino groups increase the action substantially, whereas the phenylating of the same groups has a contrary effect (rosaniline vs. methyl violet, ethyl violet, and aniline blue) (Browning and Gilmour, 1913; Kligler, 1918; Thornberry, 1931).

(4) Transforming one amine into a quaternary ammonium group greatly diminishes the action (methyl violet vs. methyl green) (Browning and Gilmour, 1913; Kligler, 1918). In our opinion this circumstance cannot be related to the reduction of the three resonating structures of methyl violet to two in methyl green, because malachite green with only two resonating structures is as active as methyl violet with three (see no. 2 above).

(5) It appears that methylated di- and triphenylmethane derivatives act against gram-positive germs chiefly, if not exclusively, in their quinoid dye form. The nonmethylated dye rosaniline, however, also is effective in the form of leucobase, although only in a lower degree (Fischer *et al.*, 1944, 1946).

Recently Kahn and Petrow (1945) published experiments on some pyridyl analogs of triphenylmethane, according to which the substitution of one or two phenyl radicals for pyridyl ones diminishes the bacteriostatic action. From our point of view one statement of these authors is of particular interest, namely, that leuco compounds of these derivatives seem to show approximately the same (low) degree of activity against gram-negative as against gram-positive organisms, whereas their oxidation products are many times more active against the latter than the former. Looking at the results of these authors one finds, furthermore, that the difference between the action of leuco compounds and that of oxidized products against gram-positive germs is less in the case of a monomethylamino derivative (1:2,000 vs. 1:256,000) than it is with dimethylamino derivative (1:2,000 vs. 1:1,024,000). This fact corresponds to our findings (1944), that there is a relatively small difference between the action of the leuco base and that of the oxidized form in the case of the nonmethylated dye rosaniline, whereas the actions of the corresponding forms of methylated dyes (gentian violet, malachite green) differ widely.

In order to explain the bacteriostatic activity of the triphenylmethane dyes, several hypotheses have been proposed. We shall consider three of them.

(1) The hypothesis of Stearn and Stearn (1923, 1924, 1926, 1930) assumes the formation of un-ionized complexes from amphoteric cell constituents and dye radicals, the basic dyes neutralizing the acidic groups of these constituents, and acid dyes the basic groups.

(2) Ingraham's hypothesis (1933) is based on a supposed poising effect of the dyes upon the oxidation-reduction potential (cf. Hoffmann and Rahn, 1944).

(3) Churchman's hypothesis (1912, 1923) is that the dyes in question saturate certain specific protoplasmic groups, which have an affinity for the former.

Stearn and Stearn point out, among other arguments, that the stronger action of basic dyes in more alkaline media, and correspondingly the stronger action of acid dyes in more acid media, are in accordance with their hypothesis. These authors explain furthermore the more potentiated bacteriostatic effects of alkylated derivatives by their increased basic character. Hence, phenylating, which lowers the basic property, diminishes the action. It is also possible to explain the importance of the quinoid structure on the same basis, such a structure being linked to the presence of an imino group of strong alkaline character.

The theory of Stearn and Stearn provides no explanation for the significance of the third phenylic ring, nor for the unimportance of the third group or the weak action of derivatives with one quaternary group, unless it can be shown that the corresponding groupings bear relationship with the ability to form un-ionized complexes.

Ingraham (1933) formulates certain objections against the exclusive validity of the hypothesis of Stearn and Stearn, some of which we shall mention below.

(1) Ingraham contradicts the statement that acid dyes act more strongly in acid media, affirming that the action of acid dyes depends only in a small degree on the pH of the media, but except for this acid dyes as well as basic dyes are invariably more effective in alkaline media.

(2) Gram-positive anaerobes are relatively dye-tolerant, as are in general those organisms which possess powerful reducing mechanisms.

Ingraham's own hypothesis was inspired by the studies of Dubos (1929), who concludes that methylene blue inhibits microbial growth by poising the oxidation-reduction potential at a point unfavorable for germ multiplication.

It seems questionable, however, whether such a conclusion can be extended to include triphenylmethane dyes, since methylene blue is a typical oxidation-reduction indicator and buffer, whereas, as Ingraham herself states, gentian violet does not significantly change the oxidation-reduction potential of bacteriological media. We can only confirm this for malachite green (Fischer *et al.*, 1944).

Otherwise, some of the arguments and experimental results of Ingraham (1933) and Hoffmann and Rahn (1944) support the assumption of an interference by gentian violet with microbial oxidation processes. Such an interference, however, does not necessarily bear any relation to a poising of the potential, but may be explained by some other mechanism, such as blocking or inactivating ferments or other biological substances (cf. Quastel, 1932; Quastel and Wheatley, 1931).

In fact, some of Ingraham's arguments and experiments intended to prove a poising effect of gentian violet are not fully convincing. Such heterogeneous processes as the simple formation of a carbinol base from the dye by NaOH and the necessarily destructive oxidation by peroxide are indistinctly designated by this author as more or less reversible "oxidation," without taking into consideration the chemical structure of the derivatives produced. The ineffectiveness of gentian violet after decolorization by iron dust and by peroxide is used, furthermore, as an argument in favor of the causal importance of changes of potential, and yet this ineffectiveness may be explained equally well as a consequence of structural alterations of very different character, such as the simple loss of quinoid structure by reduction (iron dust) and the destruction of the molecule by oxidation (peroxide).

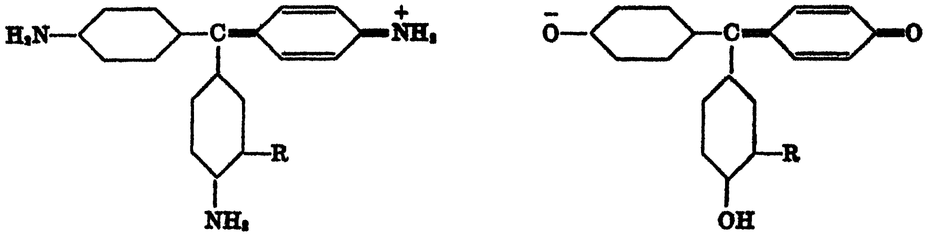
In our opinion a modernized form of Churchman's hypothesis may be accepted, namely, that triphenylmethane dyes act by blocking some important biological mechanisms, possibly connected with oxidation processes (cf. Davies, Hinshelwood, and Pryce, 1944).

EXPERIMENTAL

In the experiments to be reported here the bacteriostatic effects of rosaniline and its phenolic analog (rosolic acid) have been studied comparatively.

The commercial preparations labeled "rosaniline" or "fuchsine" consist generally of a mixture of this dye with a near homolog inaccurately called "pararosaniline" or "parafuchsine" (nor-rosaniline would be a more appropriate name), which differs from rosaniline only by the absence of a methyl group attached to one of the phenylic rings in an ortho position with respect to the amino group. The same relation exists between the corresponding phenolic analogs, rosolic acid and aurine. In order to be sure that the preparations used in our experiments corresponded to one another in every respect, we prepared our "rosolic acid" from the "rosaniline" used in these experiments according to the method of Caro and Graebe (diazotization and hydrolysis). Figure 1 shows the structures of these dyes, both of them in the form of a monovalent ion.

Rosaniline is a basic dye, which in moderately acid solutions forms a monovalent cation. The ionised group has an immonium structure (methenylquinimine ionized). Rosolic acid is a phenolic dye, which in moderately basic solutions forms a monovalent anion. The ionised group in this case is a phenol, whereas the quinoid part of the molecule is a quinone (methenylquinone).



R=H pararosaniline
R=CH₃ rosaniline

R=H aurine
R=CH₃ rosolic acid

FIGURE 1

TABLE 1

Growth of yeast in the presence of different concentrations of malachite green at varying pH values

DYE CONCENTRATION	pH					
	4.2	4.28	4.50	5.30	6.22	6.58
1:100,000	−	−	−	−	−	−
1:200,000	−	−	−	−	−	−
1:400,000	+	+	+	−	−	−
1:800,000	+	+	+	−	−	−
1:1,600,000	++	++	++	+	+	−
0	++	++	++	++	++	+

TABLE 2

Growth of yeast in the presence of different concentrations of rosaniline and rosolic acid at varying pH values

DYE CONCENTRATIONS	ROSANILINE			ROSOLIC ACID	
	pH 4.08	5.30	5.80	5.30	6.60
1:1,000	+	−	−	−	−
1:2,000	+	−	−	+	+
1:4,000	++	+	−	++	+
1:8,000	++	++	+	++	+
1:16,000	++	++	++	++	+
0	++	++	++	++	+

Besides these dyes we employed malachite green, a methylated diaminotriphenylmethane dye, with a strong bacteriostatic action. The basic dyes malachite green and rosaniline have been used in the form of chlorides and rosolic acid in the form of sodium salt. The first series of assays has been dedicated to the study of the influence of the pH of the medium upon the bacteriostatic action of malachite green against bakers' yeast. This germ is particularly interesting in this respect as it grows optimally in acid media. The yeast was cultivated in yeast water (1:8) containing 0.5 per cent glucose, buffered by acetate mixtures (M/15) and inoculated with 0.1 ml of a 1 per cent fresh yeast suspension. It was incubated for 24 hours at 20 C (table 1).

The results clearly show the dependence of the bacteriostatic action of malachite green on the pH of the medium. Similar experiments with rosaniline and rosolic acid gave the results shown in table 2.

It can be observed that the order of magnitude of the action of malachite green (table 1) is quite different from that of both rosaniline and rosolic acid (table 2), the methylated dye malachite green being effective at pH 5.3 in a dilution of 1:800,000, whereas both rosaniline and rosolic acid acted at the same pH only until they were diluted to 1:2,000 and 1:1,000, respectively. Furthermore there is clear evidence that the action of the basic dyes malachite green (table 1) and rosaniline (table 2) depends on the pH range. On the other hand, no such dependence appears in the case of the phenolic dye rosolic acid between the pH values of 5.3 and 6.6. More acid solutions could not be tested for this dye precipitated in such conditions. In more basic solutions there was no regular growth of yeast.

TABLE 3

Growth of germs in presence of varying concentrations of malachite green, rosaniline, and rosolic acid

DYES	STAPHYLOCOCCUS	YEAST	SHIGELLA	EBERTHELLA	ESCHERICHIA
Malachite green	1:8,000,000– 1:16,000,000+	1:800,000– 1:1,600,000+	1:400,000– 1:800,000+	1:80,000– 1:160,000+	1:80,000– 1:160,000+
Rosaniline	1:80,000– 1:160,000+	1:2,000– 1:4,000+	1:2,000– 1:4,000+	1:2,000– 1:4,000+	1:500– 1:1,000+
Rosolic acid	1:40,000– 1:80,000+	1:1,000– 1:2,000+	1:1,000– 1:2,000+	1:2,000– 1:4,000+	1:500– 1:1,000+
pH	7.6	5.3	7.1	7.3	7.3

In the following assays malachite green, rosaniline, and rosolic acid were tested against *Staphylococcus aureus*, *Shigella*, *Escherichia coli*, and *Eberthella typhosa*, cultivated in peptone water and in broth. Inoculation was with 0.05 ml of a 24-hour culture; readings occurred after 24 hours at 37 C (see table 3).

There appears a very close parallelism between the bacteriostatic strength of rosaniline and that of rosolic acid against all the germs studied here, which belong to very different classes of microorganisms and have very different dye sensitivities. The action of the methylated dye malachite green is in every instance of a much higher order of magnitude than that of both rosaniline and rosolic acid. It further seems that all three dyes have a stronger effect against staphylococcus than against the members of the coli-typhoid group. In the case of gram-positive germs and *Shigella*, rosaniline acts somewhat more strongly than does rosolic acid, whereas both of them act with equal strength against *Escherichia* and *Eberthella*.

DISCUSSION

It is a well-established fact that basic dyes act more strongly in more alkaline solutions, thus supporting the hypothesis of Stearn and Stearn. But apparently this hypothesis cannot be applied so easily to the action of acid dyes, as they do not act more strongly in more acid solutions, as would be required by this hypothesis. On the contrary, they behave either like the basic dyes (showing only a weaker dependence on the pH value), as Ingraham states, or they are not influenced by pH ranges at all, as observed in our assays.

To explain this discrepancy between the bacteriostatic behavior of basic and acid dyes, one may assume that the mode of action of both classes of triphenylmethane dyes is different and that the hypothesis proposed by Stearn and Stearn is only valid for basic dyes. We think, however, that the close parallelism observed between the action of rosaniline and that of rosolic acid against very different kinds of microbes makes the existence of a similar mechanism of action in both cases more probable. If this is so, the basic character would not have any fundamental importance but would represent only one of the factors able to reinforce the effectiveness.

The importance of the quinoid structure and its presence in both rosaniline and rosolic acid may be used as an argument in favor of Ingraham's thesis of poising action on the oxidation-reduction potential. However, as set forth earlier, this hypothesis is not well supported as yet. It would be necessary to determine, in any case, the potentials of the different triphenylmethane dyes and to compare the values obtained with the strength of the bacteriostatic action of the corresponding dyes, as has already been done in the case of acridine dyes by Breyer, Buchanan, and Duewell (1944; cf. Albert *et al.*, 1945).

One is impressed, furthermore, by the great difference existing between the action of alkylated dyes, on one hand, and that of a nonalkylated basic dye and an acid dye, on the other hand. Unless it can be shown that the change in the strength of the basic character of the oxidation-reduction potential caused by alkylation of the amino groups can be responsible for that difference, this circumstance may rather indicate a partially different mode of action for alkylated and nonalkylated dyes (cf. Thornberry, 1931). There seems to exist some further evidence in favor of such an assumption, for alkylation not only strengthens in a considerable degree the bacteriostatic action, but it also seems to increase the importance of the quinoid structure for this action (see earlier discussion of the varying relations between the activity of leuco bases and of quinoid dye salts in nonmethylated and methylated dyes). In pyridyl analogs of triphenylmethane, alkylating and quinoid structure are of importance solely for the action against gram-positive organisms but not for gram-negative organisms. Apparently alkylation of the amino groups alters the bacteriostatic activity not only quantitatively but also qualitatively. It seems, therefore, not only that the bactericidal and bacteriostatic effects of triphenylmethane dyes may have different mechanisms (Churchman, 1912; Hoffman and Rahn, 1944), but the mechanisms of the bacteriostatic effects of different derivatives against different microorganisms also may not be entirely identical. Finally the conclusion reached by Breyer *et al.* (1944) in the case of acridine derivatives may be valid also for triphenylmethane dyes, namely, that the activity cannot be connected to any single chemical or physical property, but represents the sum total of such properties.

ACKNOWLEDGMENT

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SUMMARY

Whereas the basic dye rosaniline acts more strongly in a more alkaline medium, the action of the phenolic (acidic) dye rosolic acid shows no dependence on the pH value.

The bacteriostatic effects of both rosaniline and rosolic acid are of the same order of magnitude, which is considerably lower than that of the methylated basic dye malachite green.

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ON THE EFFECT OF AERATION AND NUTRITION ON CELLULOSE DECOMPOSITION BY CERTAIN BACTERIA

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Microbiological decomposition of cellulosic fibers, particularly cotton, is one of the major problems confronting the Quartermaster Corps in the South Pacific. Since the nature of the organisms concerned is of primary consideration, isolations were made from 150 samples of fabrics received from the tropics, in different stages of decomposition. Of these, the bacterial isolates were studied by the writers. Of nearly 500 such isolates, 39 or roughly 8 per cent were capable of destroying cellulose. These can be divided readily into two groups: (a) a highly aerobic type, chiefly *Cytophaga* sp., and (b) a type requiring little, if any, oxygen. The latter is not truly anaerobic as it is capable of growing in the presence of oxygen, although it does not respond to differences in the oxygen concentration. In order to explore the physiology of these two groups and to determine differences in their activity on cellulose, a study of one representative isolate from each group was undertaken. In this way, it was thought that it would be possible to provide conveniently more detailed information than could be obtained from a generalized examination of many or all of the isolates. As representative of group (a) was chosen an isolate of *Sporocytophaga myxococcoides*, and of group (b) *Cellulomonas* sp.

No method capable of giving satisfactory quantitative data regarding the rate of cellulose decomposition by aerobic organisms has heretofore been reported. To be effective, a procedure must be simple enough to permit determinations on a large number of samples in a relatively short time so that many variables can be studied simultaneously. The results must be reproducible and the replicates in such close agreement that a small number will yield significant results. Finally, the method must be able to demonstrate a selective response by the organism to small changes in the environment. As will be shown in subsequent pages, a method fulfilling these conditions was found in the use of shake flasks.

PROCEDURE

The difficulties involved in a study of cellulose decomposition by aerobic microorganisms are caused primarily by the fact that cellulose is insoluble in water. A satisfactory substitution for solution can be obtained, however, by mechanically or chemically reducing the cellulose to the finest particles that still possess the properties of the original material.

Approximately 4 g of ground filter paper was placed in a Waring "blendor," and the desired mineral salts solution was added. After 2 minutes of agitation the suspension was transferred to a flask and made up to 1,000 ml with the same solution. Twenty-five-ml aliquots were placed in 250-ml Erlenmeyer flasks, which were plugged with cotton and sterilized. Substances to be tested were then introduced in sterile 1-ml portions, after which the flasks were inoculated with 0.25 to 0.50 ml of a suspension of the organism. The accompanying uninoculated control flasks were used for determinations of the original amount of cellulose and for the original determinations of hydrogen ion concentration.

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The mineral solution selected was that of Fuller (1942) containing 0.1 per cent $NaNO_3$, 0.1 per cent K_2HPO_4, 0.05 per cent $MgSO_4 \cdot 7H_2O$, 0.05 per cent KCl, 0.003 per cent yeast extract Difco (in place of yeast water), and 0.4 per cent ground filter paper. Twenty-five ml of the suspension per flask was found convenient.

After a period predetermined for each organism, the flasks were removed from the shaker. The pH of the contents was determined by means of a Beckman pH meter. Two and one-half ml of N/1 KOH was then added to the contents, and the flask was autoclaved for 15 minutes at 15 pounds' pressure. The residue was filtered while hot through a Gooch crucible, washed with water, and dried at 102 C for 16 hours. After cooling, the crucibles were weighed, ignited in a muffle furnace, and reweighed. The uninoculated controls therefore represent the original weight of *ash-free* cellulose, and the differences (control minus test) are losses in organic matter. Since no correction was made for the weight of the bacteria, the actual percentage of loss in *cellulose* was always greater than that reported.

Filtration. One of the first problems in technique to be solved was that of filtering. It was soon observed that at least 72 hours was required to filter the 25 ml of residual mixture in the flasks after incubation. In order to speed up this operation several methods were tried, the KOH heat treatment described above proving most satisfactory By this method, the time required for filtering was reduced to 5 to 15 minutes, although an occasional sample required up to 30 minutes. The effect of the 0.1 N KOH and autoclaving on various substrates is shown in table 1.

The results reveal only slight decomposition of the original cellulosic substrates, except for the cellulose dextrin with which the KOH treatments result in a partial dissolution. The effect of alkali on the residue is much greater than on the pure substrate. It is believed that this is a result of the decomposition of bacteria and bacterial mucilage by the treatment, although the possibility exists that intermediates of cellulose, similar to the dextrins described above, are removed. In either case, it is thought that such treatment, in addition to speeding up the filtering, gives a closer approximation to the actual amount of cellulose decomposed.

Fuller (1942) also used an alkali treatment, but without heat, and followed by 1 per cent acetic acid. His purpose was not, however, related to the problem of filtration described above.

TABLE 1

Effect of prefiltering treatment with KOH on loss in weight of cellulose and of cellulose residues

SUBSTRATE	% LOSS IN WEIGHT DUE TO KOH + AUTOCLAVING
Cellulosic material	
Filter paper, ground	0
Cotton fabric	3
Cellulose dextrin (from H_2SO_4)	78*
Residues filter paper—	
7 day decomposition by *S myxococcoides*	10
11-day decomposition by *S myxococcoides*	21
7-day decomposition by *Cellulomonas* sp	8
7 day decomposition by *Spirochaeta cytophaga*, Gray's USDA	11
11 day decomposition by *Spirochaeta cytophaga*, Gray's USDA	16

* Without KOH there was a 38 per cent loss on autoclaving

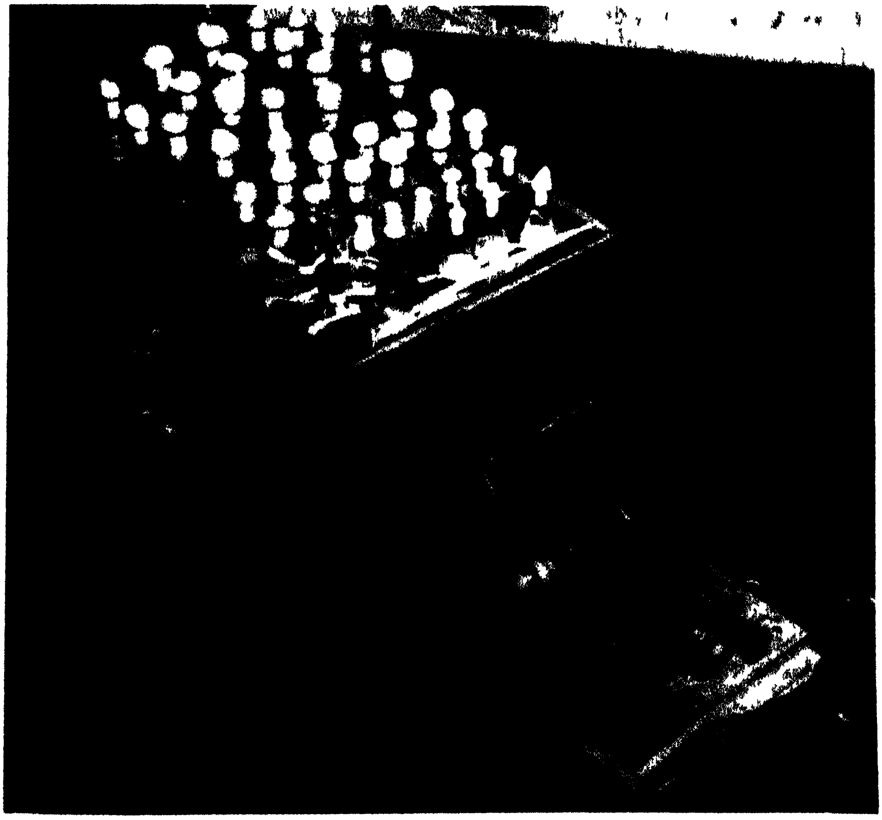

FIG 1. SHAKER MODIFIED TO GIVE 120 CYCLES PER MINUTE

A definite contribution to the problem of aerobic decomposition of cellulose was made by Fuller and Norman (1943) when they bubbled air through large volumes of a filter paper suspension in order to obtain quantities of residue for chemical analysis. Since our objective was to devise a method for studying the optimal conditions for decomposition, it was possible to decrease the volume of suspension to 25 ml, aerated in 30-ml micro-Kjeldahl flasks. Several experiments employing this technique proved the method to be entirely adequate, but difficulties of manipulation (foaming, evaporation, sterility, and aeration rate) made the method less desirable than the shaking technique subsequently used.

TABLE 2

Effect of flask size on decomposition of filter paper on a shaker doing 120 cycles per minute

ORGANISM	% LOSS IN WEIGHT*		
	Shaken flasks		Unshaken
	125 ml	250 ml	250-ml flask
Sporocytophaga myxococcoides	54	54	13
Cellulomonas sp.	65	58	32

* Seven-day incubation period; 25 ml of medium.

TABLE 3

Effect of flask size on decomposition of filter paper by Cellulomonas sp.

FLASK SIZE	SUSPENSION	DEPTH	AVERAGE % LOSS IN WEIGHT*	
			Open flasks†	Sealed flasks‡
ml	*ml*	*mm*		
250	25	5	14	—
125	25	9	19	19
125	50	16	24	21
50	25	15	34	27

* Six-day incubation period.
† Cotton stoppers.
‡ Sealed with paraffin.

AGITATION BY SHAKE FLASKS

The speed of the reciprocal motion box shakers commonly available is much too high for use in bacteriological work. Reduction of the speed to 120 complete strokes per minute has been found satisfactory with 125-ml and 250-ml flasks.

Since 100 mg of ground filter paper in 25 ml of mineral salts solution was found to be the maximum amount if filtering was to be completed in a reasonable time, this quantity was used in the flasks on the shaker. Four replicates were used for each variable. The shaker was stopped for a 4-hour period each day, and each flask was swirled gently to remove any ring which may have formed on the sides of the flask. Incubation was at 30 C, though temperatures sometimes went slightly above that. At the completion of each experiment, the residue was treated as described above.

The effect of flask size on the rate of cellulosic decomposition by the two organisms was first studied.

With *S. myxococcoides*, shaking increased the rate of decomposition fourfold, whereas with *Cellulomonas* sp. the rate was merely doubled. The former is, therefore, a much more aerobic organism than the latter. The odd position which the *Cellulomonas* sp. occupies is made clear from table 3. As the depth of the suspension increases, the rate of decomposition increases. But sealing lowers the rate, the inhibition increasing with decreasing air volume of the flask. It appears that abundant air retards decomposition by this organism. Erlenmeyer flasks of 125- or 250-ml volume are adequate for *S. myxococcoides*, but the 50-ml size is superior for decomposition by *Cellulomonas* sp.

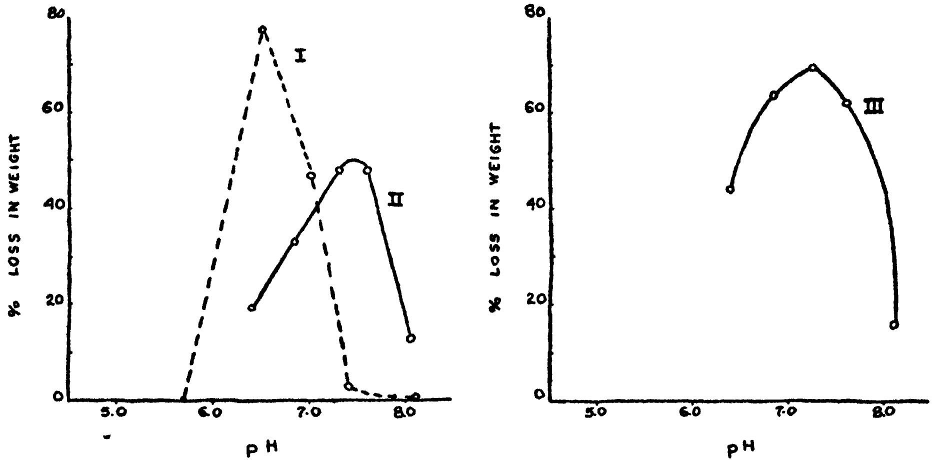

FIG. 2. EFFECT OF pH ON DECOMPOSITION OF FILTER PAPER IN SHAKE FLASKS BY MICROORGANISMS

Curve I, *S. myxococcoides* (4 days); curve II, *Cellulomonas* sp. (6 days); curve III, *Actinomyces* sp. (PQD-B36D) (6 days).

EFFECT OF HYDROGEN ION CONCENTRATION

The effect of pH on decomposition may be related to the nature of the medium. In the present experiments, the medium for *S. myxococcoides* was a mineral salts solution containing 12 ppm iron and no organic matter but the cellulose. The medium for the other organisms contained, besides the salts, urea as a source of nitrogen, yeast extract, and gelatin. In all cases, the optima for cellulose decomposition fall within the pH range 6.5 to 7.5.

EFFECT OF NITROGEN SOURCE

In studying the effect of the nitrogen source on the rate of decomposition, two nitrates, two ammonium compounds, and urea were selected (table 4). The nitrogen source was added in quantity equivalent to 0.165 g nitrogen per liter.

For both organisms ammonium nitrogen is as good as nitrate nitrogen, but all ammonium compounds are not of equal value. The effect of the anion is quite definite, the carbonate being superior to the sulfate, though the difference in pH may be the deciding factor. As the ammonium nitrogen is utilised, the pH drifts, the drift to the acid side being much greater for *Cellulomonas* sp. (pH 4.7) than for the *S. myxococcoides* (pH 6.2). It may be that urea is a good nitrogen source for *Cellulomonas* sp. because there is so little change in pH during its utilization.

TABLE 4

Effect of nitrogen source on rate of decomposition of cellulose by S. myxococcoides and Cellulomonas sp.

NO.	NITROGEN SOURCE	*S. myxococcoides* 4 days			*Cellulomonas* sp. 6 days		
		pH		Avg % loss*	Orig.	Final	Avg % loss* in weight
		Orig.	Final				
A	$NaNO_3$	6.8	7.7	66	7.3	8.0	46
B	$Mg(NO_3)_2$	6.4	7.7	65	6.8	7.5	48
C	$(NH_4)_2CO_3$	6.9	5.5	61	7.7	5.0	43
D	$(NH_4)_2SO_4$	6.5	6.2	25	6.8	4.7	27
E	Urea	6.9	7.2	3	7.3	6.7	68

* Average of 4 replicates.

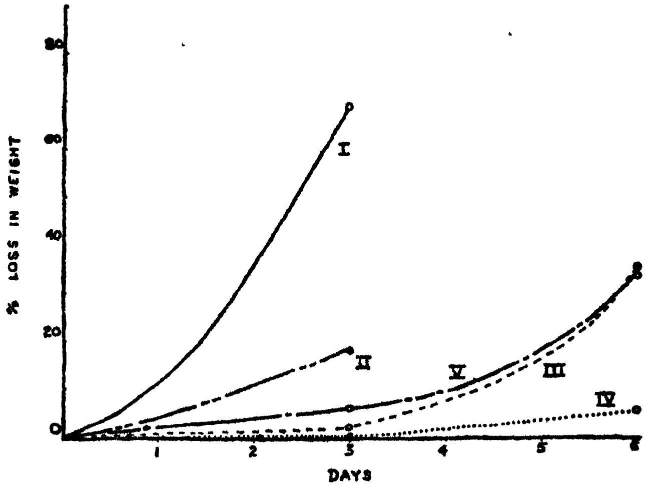

FIG. 3. EFFECT OF UREA CONCENTRATION ON RATE OF DECOMPOSITION BY S. MYXOCOCCOIDES (IN THE PRESENCE OF NITRATE N)

I = no urea; II = 0.05 g N per liter as urea; III = 0.14 g N per liter as urea; IV = 0.41 g N per liter as urea; V = IV plus *E. coli* inoculum.

Urea exerts an interesting influence on these organisms. In the case of *S. myxococcoides*, not only is it not available (table 4), but it is actually toxic (figure 3). When it is added to the usual medium containing $NaNO_3$, it considerably reduces the rate of decomposition. Winogradsky (1939) reported a similar inhibition by 0.3 M urea in the case of another organism, *Nitrobacter*. Our results show a much higher degree of toxicity, 0.002 M urea slowing down the rate significantly. A light *Escherichia coli* inoculum added to the solution of highest urea concentration increases the rate of decomposition over that shown without the *E. coli*, apparently by reducing the urea concentration from 0.41 to approximately 0.14 g N per liter. (This high degree of toxicity of urea does not apply to its use in agar, where fabric strips are being decomposed on the agar surface.)

In direct contrast with the foregoing is the effect of urea on *Cellulomonas* sp. (table 4). This bacterium prefers urea nitrogen. The third possibility, urea nitrogen being equal to nitrate nitrogen, is exemplified by *Actinomyces* sp. (PQD-B36D). At the end of 6 days' incubation on the shaker, the medium containing urea gave 59 per cent loss in weight while that containing sodium nitrate also gave 59 per cent loss.

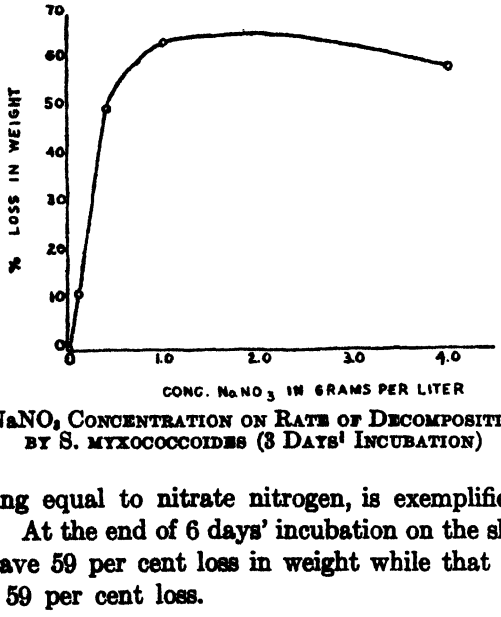

FIG. 4. EFFECT OF $NaNO_3$ CONCENTRATION ON RATE OF DECOMPOSITION OF FILTER PAPER BY S. MYXOCOCCOIDES (3 DAYS' INCUBATION)

EFFECT OF OTHER SALTS

Examination of the value of other salts in the medium indicated that for both bacteria potassium chloride had practically no effect up to a concentration of 2.5 g per liter. Above this concentration, a toxic action is apparent (figure 5), which is probably a result of the high total salt concentration. When studying the action of a particular salt, therefore, it would appear to be necessary to vary the concentration of the other salts, so that the total molar concentration remains constant. This is especially important when the concentration being studied exceeds 0.06 N. Above that point, the effects of the variable would be obscured by the high total concentration.

Magnesium sulfate is important to both bacteria. The addition of 0.5 g per liter of this salt nearly doubled the rate of decomposition. Iron salts are stimulatory to *S. myxococcoides* but show no such effect on *Cellulomonas* sp. (figure 6). If, however, the phosphate concentration is increased from 0.01 M to 0.06 M, the stimulatory effect of iron on *S. myxococcoides* disappears.

Results with other minor elements (Cu, Zn, B, Mo, Mn) show no stimulation whatsoever. Copper is toxic[2] to both organisms at 1 ppm. Manganese is toxic to *Cellulomonas* sp. at 1 ppm but is without effect on *S. myxococcoides*, even at 5 ppm.

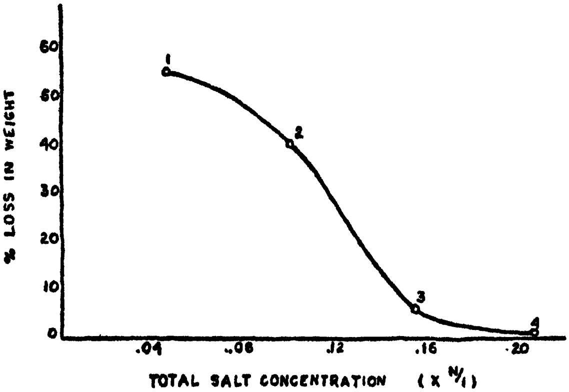

FIG. 5. EFFECT OF INCREASING POTASSIUM CHLORIDE CONCENTRATION ON DECOMPOSITION OF FILTER PAPER BY S. MYXOCOCCOIDES (3 DAYS)

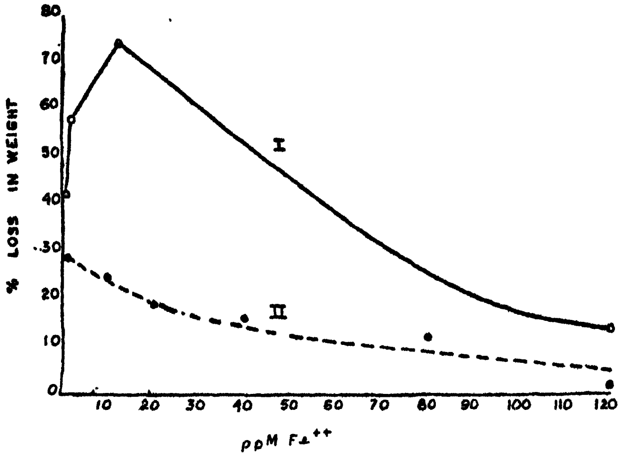

FIG. 6. EFFECT OF IRON CONCENTRATION ON RATE OF DECOMPOSITION OF FILTER PAPER BY MICROORGANISMS
Curve I, S. myxococcoides (5 days); curve II, Cellulomonas sp. (6 days).

EFFECT OF YEAST EXTRACT AND GELATIN

In the aeration experiments, there was an indication that yeast extract and gelatin might be of some value to *S. myxococcoides* in aerated flasks. Since only 12 per cent decomposition was obtained in 6 days, however, shaker tests (table 5) were run to clarify the issue. The results indicate that no advantage is gained by the addition of these substances to the medium found optimum for this organism. On the other hand, results with *Cellulomonas* sp. on the shaker (table 6) confirm those of the aeration tests regarding the necessity of yeast and of gelatin for maximum decomposition.

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[2] By toxic here is meant a decrease in the rate of decomposition from about 50 per cent for controls to less than 5 per cent for flasks containing the element in question.

TABLE 5

Effect of yeast extract and gelatin on cellulose decomposition by S. myxococcoides in shaker flasks

NO.	VARIATION		MG RESIDUE					AVG % LOSS IN WEIGHT	pH	
	% gelatin	% yeast extract	1	2	3	4	Avg		Orig.	Final
1	0	0	24	27	29	33	28	71	7.2	7.9
2	0	0.004	26	27	27	30	28	71	7.2	7.9
3	0	0.04	31	32	37	—	33	66	7.2	7.9
4	0.004	0.004	26	29	30	30	29	70	7.2	7.9
5	0	0	24	29	31	—	28	71	7.2	7.9
6	Uninoc. controls		94	96	96	99 97	94.4	—	—	—

TABLE 6

Effect of yeast extract and gelatin on decomposition of filter paper by Cellulomonas sp. (6 days on shaker)

NO.	VARIATION		MG RESIDUE					AVG % LOSS IN WEIGHT	pH		
	% gelatin	% yeast extract	1	2	3	4	Avg		Orig.	Final	
1	0	0	102	102	99		101	0	7.5	7.2	
2	0	0.004	75	75	78		76	25	—	7.4	7.5
3	0.004	0.004	71	74	67		71	30	7.5	7.5	7.7
4	0.004	0.04	35*	37	35		36	64	7.5	6.5	
5	0.04	0.004	79	74	72		75	26	7.5	6.3	6.4
6	Uninoc. checks		101	102	99	100	101	—	—	—	

* Five-day result.

DECOMPOSITION RATE AT OPTIMAL CONDITIONS

Under conditions believed to be optimal for each organism, the rate of decomposition of cellulose in shaker flasks was determined. For S. *myxococcoides* the medium contained M/1 potasium phosphate buffer (pH 6.7), 10 ml; $NaNO_3$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.05 g; and the cellulose at 4 g per liter. For *Cellulomonas* sp., the medium consisted of urea, 0.356 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; M/1 potassium phosphate buffer (pH 7.6), 10 ml; yeast extract, 0.3 g; gelatin, 0.03 g; and ground filter paper, 4 g per liter. The first medium was placed in 250-ml flasks, and the second in 50-ml flasks. The cultures were removed from the shaker at various intervals, and the loss in cellulose was determined. The results of this experiment are plotted in figure 7.

Cellulomonas sp. decomposed filter paper at a rate equal to that of *S. myxococcoides*. The figure, 50 per cent loss in weight in 3 days, is, however, not equal to the best obtained by the latter. It is the highest rate obtained by the *Cellulomonas* sp. in any of the present experiments.

All curves finally level off while the undissolved residue remaining is still over 20 per cent. Just how much of the residue is bacterial substance and how much cellulose has not been determined.

With *Cellulomonas* sp., there was a continued drop in pH. Thus, the final pH values in either case were near the extreme end of the range of activity for each organism, so that any remaining cellulose would be decomposed at a very slow rate. Calcium carbonate added to three flasks of *Cellulomonas* sp. at the beginning of the experiment kept the pH up to 6.8 to 7.0 at the end of 5 days (compared to 6.1 without calcium carbonate), but the amount of final residue (36 mg) was not significantly different from that without calcium carbonate (33 mg). It appears, therefore, that the residue is mainly bacterial substance and not cellulose.

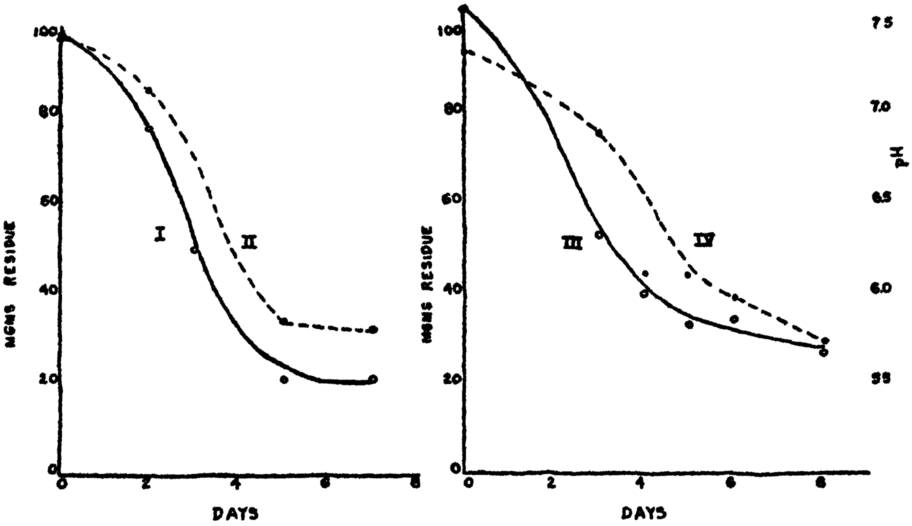

FIG. 7. RATE OF DECOMPOSITION OF CELLULOSE
S. myxococcoides, curve I, filter paper; curve II, cotton cloth.
Cellulomonas sp., curve III, filter paper; curve IV, pH vs. time.

It is believed that the two organisms studied, isolated from fabric samples, should prove useful in tests designed to determine the resistance of treated cotton fabric to microbial action. It is of the utmost importance that organisms showing the greatest *dissimilarity* be used in such tests. *S. myxococcoides* and the fungi are alike in many respects. They are highly aerobic and show a preference for low pH, but they differ in mode of action on the fiber. *Cellulomonas* sp. differs from all in being most active as anaerobic conditions are approached.

APPLICABILITY OF SHAKER METHOD TO FUNGI

To this point, data have been presented relative to the use of aeration and particularly of shaker flasks, in a study of cellulose-decomposing bacteria. The applicability to fungus studies was next investigated. Five fungi of different cellulose-decomposing ability were selected. *Aspergillus niger* (J745) was included because of conflicting reports on its ability to utilize cellulose. The other fungi are recognized cellulose destroyers.

Humicola, *Metarrhizum*, and *Chaetomium* formed heavy rings of deposited material on the flask above the liquid level. These were not washed down during the course of the experiment. At the end of the incubation period, the residues were treated with 0.1 volume M/1 potassium hydroxide and autoclaved, as was customary with the bacterial residues. In spite of the ring formation, the results of duplicate flasks were in good agreement, and the rate of breakdown was rapid. Furthermore, the descending order of activities (*Humicola* and *Metarrhizum*, *Chaetomium*, *Aspergillus terreus*, *A. niger*) is the same as that determined for these organisms by Dr. W. L. White, of the Tropical Deterioration Research Laboratory, using the loss in tensile strength of fabric on agar as a criterion. *A. terreus*, which was cultured for 6 days, formed less of a ring, and the ring was washed down daily. This would tend to result in more uniform action than would be found with the heavy rings of the faster-growing fungi.

TABLE 7

Decomposition of filter paper in shaker flasks by various fungi

FUNGUS	DAYS INCUBATED	MG RESIDUE			AVG % LOSS IN WEIGHT
		1	2	Avg	
Chaetomium globosum, USDA 1042.4	3	90	89	90	14
Humicola sp., PQMD 34e	3	50	56	53	50
Metarrhizum glutinosum, USDA 1334.2	3	59	64	62	41
Uninoc. controls	3	103	105		—
		105	107	105	
Aspergillus terreus, MIT 7	6	52	49	51	51
Aspergillus niger, J745	6	104	106	105	0

SUMMARY

Two methods for studying cellulose decomposition quantitatively were investigated. Both methods gave good results. Aeration involved the problem of preventing contamination and wetting plugs by bubble formation. The use of shaker flasks at 120 cycles per minute was shown to be an excellent method for the study of microbial decomposition of cellulose.

The optimal conditions for two cellulose-decomposing organisms were partially worked out. It was shown that *S. myxococcoides* is a strongly aerobic organism,

capable of high rates of decomposition of pure cellulose. It is greatly stimulated by 10 ppm Fe^{++} and has its optimal pH near 6.5. No growth factors appear to be necessary. *Cellulomonas* sp. requires very little oxygen for its optimal rate of decomposition but is dependent upon an external source of growth substances. It is not stimulated by iron, has an optimal pH near 7.5, and utilizes urea nitrogen preferentially to nitrate nitrogen.

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COMPARATIVE ACTION OF BROMINE AND IODINE ON TOXIC ENZYMES OF STAPHYLOCOCCUS AUREUS AND STREPTOCOCCUS PYOGENES[1]

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It is well known that iodine is widely used as a disinfectant for small wounds. The aim of the present work was to find out whether bromine can replace iodine for this purpose.

Experiments carried out by Babcock (1945) on man and by the author on rabbits *in vivo* (to be published shortly) showed that treatment of wounds with elementary bromine had a marked therapeutic effect. Toxicity tests comparing the action of bromine and iodine on leucocytes and living embryos, the results of which will be published shortly, have shown further that bromine is a good disinfectant *in vivo*, and that it is even more effective than iodine in these conditions.

In order to understand these results better it was decided to investigate the effect of these two halogens on a number of bacterial toxic enzymes, viz., hemolysins, fibrinolysins, coagulase, and spreading factor. Tests of these agents were held to be of interest because it seemed possible that the inhibition of their action by antiseptics is connected with the activity of the latter when applied *in vivo*. Destruction of the enzymes would prevent the microorganisms from damaging the attacked body.

METHODS AND RESULTS

The effect of bromine and iodine on the coagulase of Staphylococcus aureus.[2] Citrated rabbit's blood was centrifuged for 5 minutes. The plasma obtained was diluted 1:10; 0.3 ml of the latter were added to 0.5 ml of filtrate obtained from a *Staphylococcus aureus* culture grown in broth for 24 hours at 37 C. The broth was filtered through a Berkefeld N filter. After the contents had been well mixed, the test tubes were incubated at 37 C until a clot was detected.

Varying concentrations of bromine and iodine in distilled water were added to the filtrate containing the coagulase. Then plasma was added, and the whole was incubated for 24 hours at 37 C. The concentrations of halogens which inhibited the formation of the clot were determined. Bromine in a concentration

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[1] Based on data submitted in partial fulfillment of the requirements for the degree of Ph.D., The Hebrew University, January, 1946.

The investigation was supported by a grant from Palestine Potash, Ltd.

[2] An attempt was made to reduce bromine and iodine to bromide and iodide, respectively, after different contact times between halogen and enzyme. Potassium iodide and sodium thiosulfate were used for this purpose. However, complications were encountered in these experiments as the tetrathionate which is formed by the reaction has an inhibiting effect on the enzymes. The procedure was therefore abandoned.

of 0.3 per cent or more prevents the appearance of the clot. With iodine, the same inhibition is caused only in a concentration of at least 0.8 per cent or more. Thus, bromine is 3.5 times as active as iodine as a suppressor of the coagulase of *S. aureus.*

The effect of bromine and iodine on the coagulase of Streptococcus pyogenes. Undiluted plasma, obtained from citrated rabbit's blood, was used. Three-tenths ml of plasma were added to 0.3 ml of supernatant fluid, which was obtained by the centrifugation of *Streptococcus pyogenes* grown in broth containing 3 per cent serum for 24 hours at 37 C. After thorough mixing, incubation lasted 24 hours, and at the end of this time the results were read.

TABLE 1

The action of coagulase obtained from Staphylococcus aureus and Streptococcus pyogenes in the presence of iodine and bromine

HALOGEN TESTED	CONCENTRATION IN PER CENT	SOURCE OF COAGULASE	COAGULATION AFTER TIME OF CONTACT BETWEEN COAGULASE AND HALOGEN (MIN)				
			5	15	30	60	90
I_2	0.9	*S. aureus*	−	−	−		
	0.8	*S. aureus*	−	−	−		
	0.7	*S. aureus*	+	+	+		
	0.6	*S. aureus*	+	+	+		
Br_2	0.4	*S. aureus*	−	−	−		
	0.3	*S. aureus*	−	−	−		
	0.2	*S. aureus*	+	+	+		
	0.1	*S. aureus*	+	+	+		
−	−	*S. aureus*	+	+	+		
I_2	0.6	−	−	−	−		
Br_2	0.1	−	−	−	−		
I_2	0.05	*S. pyogenes*			−	−	−
	0.04	*S. pyogenes*			−	−	−
	0.03	*S. pyogenes*			+	+	+
	0.02	*S. pyogenes*			+	+	+
Br_2	0.3	*S. pyogenes*			−	−	−
	0.2	*S. pyogenes*			−	−	−
	0.1	*S. pyogenes*			+	+	+
	0.09	*S. pyogenes*			+	+	+
−	−	−			+	+	+
I_2	0.01	−			−	−	−
Br_2	0.09	−			−	−	−

The method used for testing the coagulase of the staphylococcus was employed with the streptococcus. As is seen in the second part of table 1, bromine in a concentration of 0.2 per cent or above suppresses the action of coagulase. Iodine was more effective than bromine since in a concentration as low as 0.04 per cent it inhibited the action of the coagulase. Thus the activity ratio is 5 in favor of iodine.

The effect of bromine and iodine on staphylolysin and streptolysin. S. aureus was grown in broth constantly aerated. *S. pyogenes,* on the other hand, was not aerated during growth, but 3 per cent of rabbit's serum was added to the broth. After growth for 24 hours at 37 C, the broth was centrifuged for 25 minutes. The supernatant fluid which contained the hemolysins was removed. The broth containing the bacteria was not used, as it was important to eliminate the effect of the halogens on the bacteria themselves in order to determine the effect upon the hemolysins per se. Equal amounts of a suspension of 1 per cent washed rabbit's erythrocytes in saline were added to the solution of the hemolysins. The results of hemolysis were read after this mixture had been kept for 2 hours at 37 C and for 24 hours in an icebox.

TABLE 2

Action of staphylolysin and streptolysin in the presence of iodine and bromine

HALOGEN TESTED	CONCENTRATION IN PER CENT	LYSIN ADDED	HEMOLYSIS AFTER TIME OF CONTACT BETWEEN LYSIN AND HALOGEN (MIN)			
			2.5	5	7.5	10
		ml				
I_2	0.5	Staphylolysin 0.5	−	−	−	−
	0.4	Staphylolysin 0.5	±	±	±	−
	0.3	Staphylolysin 0.5	+	+	+	+
	0.2	Staphylolysin 0.5	+	+	+	+
Br_2	0.09	Staphylolysin 0.5	−	−	−	−
	0.075	Staphylolysin 0.5	±	±	±	±
	0.06	Staphylolysin 0.5	+	+	+	+
	0.05	Staphylolysin 0.5	+	+	+	+
−	−	Staphylolysin 0.5	+	+	+	+
I_2	0.3	−	−	−	−	−
Br_2	0.06	−	−	−	−	−
I_2	0.2	Streptolysin 0.5	−	−	−	−
	0.1	Streptolysin 0.5	±	−	−	−
	0.08	Streptolysin 0.5	±	±	±	±
	0.07	Streptolysin 0.5	+	+	+	+
Br_2	0.04	Streptolysin 0.5	−	−	−	−
	0.03	Streptolysin 0.5	−	−	−	−
	0.02	Streptolysin 0.5	±	±	±	−
	0.01	Streptolysin 0.5	+	+	+	+
−	−	Streptolysin 0.5	+	+	+	+
I_2	0.08	−	−	−	−	−
Br_2	0.01	−	−	−	−	−

Dilutions of bromine and iodine in distilled water in concentrations of 0.5 to 0.01 per cent were mixed with the supernatant fluid and left in contact for 2½, 5, 10, and 30 minutes. The minimum concentrations of halogens that destroy the hemolytic power of the fluid were determined.

It was found that bromine in a concentration of 0.075 per cent destroyed the hemolytic power of the staphylolysin, whereas iodine exerted the same effect in a concentration of 0.4 per cent or above. In the case of streptolysin it was found that bromine in a concentration of 0.02 per cent destroys the activity, whereas iodine exerts the same effect only in concentrations of 0.08 per cent or above.

Thus it seems that bromine has 5.3 times the activity of iodine in destroying the hemolysins of *S. aureus* and 4 times the activity of iodine with respect to the hemolysins of *S. pyogenes*.

The effect of bromine and iodine on fibrinolysin of S. pyogenes (group A). According to Tillett (1938) a striking liquefaction of fibrin is obtained when the source of bacteria and fibrin is from the same species, human or animal.

Fibrinolysin from *S. pyogenes* from a human source and the plasma from human blood were used. The bacteria were grown in broth containing 3 per cent serum for 24 hours at 37 C. The broth was then centrifuged for 25 minutes (2,500 rpm). The supernatant fluid containing the fibrinolysin, but no bacteria, was removed and used. Three-tenths ml of the latter fluid were added to 0.3 ml of citrated human plasma previously diluted 1:5 in saline. Then 0.2 ml of 0.25 per cent $CaCl_2$ were added, thoroughly mixed, and incubated at 37 C. After the appearance of a clot the test tubes were removed to 45 C for another 24 hours. The results were then read.

TABLE 3

Action of fibrinolysin obtained from Streptococcus pyogenes in the presence of iodine and bromine

HALOGEN TESTED	CONCENTRATION OF HALOGEN IN PER CENT	QUANTITY OF FIBRINOLYSIN ADDED	FIBRINOLYSIS AFTER TIME OF CONTACT BETWEEN FIBRINOLYSIN AND HALOGEN (MIN)		
			30	60	90
		ml			
I_2	0.06	0.3	–	–	–
	0.05	0.3	–	–	–
	0.04	0.3	–	–	–
	0.03	0.3	+	+	+
	0.02	0.3	+	+	+
Br_2	0.06	0.3	–	–	–
	0.05	0.3	+	+	+
	0.04	0.3	+	+	+
–	–	0.3	+	+	+
I_2	0.03	–	–	–	–
Br_2	0.04	–	–	–	–

Equal amounts of the fluid containing fibrinolysins were added to various concentrations of the halogens. The exposure was between 30 to 90 minutes. The concentrations which prevent the appearance of liquefaction were determined. Bromine and iodine are about equal in their inhibitory activity on fibrinolysin.

The effect of bromine and iodine on spreading factors. The spreading factor is an enzyme found in many bacteria. The penetrating power of the microorganisms through tissues is probably due to this enzyme (Duran-Reynals, 1933, 1935, 1942). The spreading factor of testicular extracts was used as it is known to be identical with the spreading factor obtained from bacteria.

Bulls' testes were stripped of membrane. The glandular tissue was ground with sand and 4 volumes of isotonic saline. The extract was centrifuged and then filtered through a Berkefeld V or W filter. The fluid can be stored in an icebox for months. For testing the effect of spreading factor the solution was diluted with equal parts of saline, and 0.2 ml were mixed in a syringe with 0.3 ml of a diluted solution of india ink (one drop per 10 ml of saline). Afterwards this mixture was injected intracutaneously in a rabbit. Control injections without the spreading factor were conducted on the same rabbit. The area through which the fluid spreads was measured after specified time intervals.

Various dilutions of bromine and iodine were added in equal amounts to the spreading factor in dilutions as described above. The range of contact varied from 2 minutes to 24 hours at room temperature. After the contact, 0.5 ml of the mixture were injected intracutaneously and the blackened area was measured after 30 minutes.

TABLE 4

Action of the spreading factor brought in contact with halogens at least for 2 minutes
(Area spread measured 30 minutes after injection)

Br_2	AREA SPREAD	I_2	AREA SPREAD
per cent	cm^2	*per cent*	cm^2
1.0	0.5–0.7	1.0	0.7
0.5	0.5–0.7	0.9	0.7
0.3	0.5–0.7	0.8	0.7
0.2	4–5	0.75	4–5
Control	4–5	Control	4–5

TABLE 5

Minimum suppressive concentrations of bromine and iodine for different toxic enzymes

ENZYME	SOURCE	MINIMAL ACTIVE CONCENTRATION OF HALOGEN	
		Br_2	I_2
		per cent	*per cent*
Coagulase	*S. aureus*	0.3	0.8
Coagulase	*S. pyogenes*	0.2	0.04
Hemolysin	*S. aureus*	0.075	0.4
Hemolysin	*S. pyogenes*	0.02	0.08
Fibrinolysin	*S. pyogenes*	0.06	0.04
Spreading factor	Bulls' testes	0.2	0.75

It was found that the inhibitory concentrations were as effective after 2 minutes as after 24 hours. Concentrations which failed to inhibit the action after a contact of 2 minutes did not show any influence after a contact of 24 hours.

It was found that bromine in a concentration of 0.2 per cent was able to eliminate the action of the spreading factor, whereas iodine exerted the same effect only in a concentration of 0.75 per cent or more. Bromine was therefore 3.75 times as active as iodine.

ACKNOWLEDGMENT

The author wishes to state that this investigation owes much to the interest and help of her late teacher Professor I. J. Kligler.

SUMMARY

Minimum suppressive doses of bromine and iodine for different toxic enzymes of pathogenic cocci have been determined.

The coagulase of *Staphylococcus aureus* was attacked and destroyed by bromine at a concentration of 0.3 per cent, whereas iodine had the same effect only at a concentration of 0.8 per cent.

With the coagulase of *Streptococcus pyogenes* iodine in a low concentration of 0.04 per cent had the same effect as bromine in a concentration of 0.2 per cent.

For the hemolysins of *S. aureus* it was found that bromine in a concentration of 0.075 per cent destroyed the activity, whereas iodine exerted the same effect only in a concentration of 0.4 per cent.

Hemolysins of *S. pyogenes* were also more readily attacked by bromine than by iodine. The former stopped hemolysin action in a concentration of 0.02 per cent, whereas the latter was effective only in a concentration of 0.08 per cent.

Fibrinolysin of *S. pyogenes* was eliminated by bromine in a concentration of 0.06 per cent and by iodine in a concentration of 0.04 per cent.

The spreading factor was destroyed by bromine in a concentration of 0.2 per cent, whereas iodine had to be used in a concentration of 0.75 per cent in order to exert the same effect.

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THE RELATIONSHIP BETWEEN LACTOBACILLUS ACIDOPHILUS (MORO) HOLLAND AND LACTOBACILLUS CASEI (ORLA-JENSEN) HOLLAND[1]

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The identification of the various species of the genus *Lactobacillus* presents a problem in that so many characters of the species in the genus as a whole are similar. Characters such as the utilization of carbon compounds are variable among the strains of a species as well as among the species and, therefore, are valuable only when correlated with other characters. Observations over a period of years have led me to wonder whether character variations might not be due to change or dissociation of strains, and therefore some of the data have been reviewed from this viewpoint.

In the original description of *Lactobacillus acidophilus* (*Bacillus acidophilus*) by Moro (1900) he described a slender gram-positive rod which formed small colonies of irregular shape with numerous fine radiant or ramified projections. The organism produced a weak acid reaction in milk. Kulp and Rettger (1924) and Curran, Rogers, and Whittier (1933) found that the species produced inactive lactic acid. Von Freudenreich (1897) isolated several acid-producing bacteria from milk products and designated them by Greek letters. Orla-Jensen (1919) designated the strains *Bacillus* α and ε of von Freudenreich as *Streptobacterium casei* and *Thermobacterium helveticum*. Orla-Jensen stated, in discussing the species *Streptobacterium casei*, "The power of forming dextro-lactic acid is, however, by far the most constant, and many strains which at first formed almost exclusively inactive lactic acid have yet in the course of years ended by forming pure dextro-lactic acid." In a footnote he further stated: "They often exhibit exactly the same mutations. They may, however, also be found to differ suddenly in their relation to one or another of the sugars." The significance of these statements was not appreciated until recently. Kopeloff and Kopeloff (1937) observed that strains of *Lactobacillus acidophilus* that produced rough colonies produced inactive lactic acid, whereas strains that produced smooth colonies produced dextrorotatory lactic acid. Hadley, Bunting, and Delves (1930) believed they could convert colony types by selection.

The form of lactic acid produced by bacteria has been used to characterize types since the early work of Schardinger (1890) and Nencki (1891), who reported that pure cultures always produce the same type of acid. Most of the late work upon strains of lactobacilli has shown that a species will always produce the same type of acid. Curran, Rogers, and Whittier (1933) studied a number of strains which had been identified by various workers as *Lactobacillus acidophilus*. Many of them produced inactive lactic acid, but some produced dextrorotatory lactic acid. In studying strains of *Lactobacillus plantarum*, Pederson (1936) found that the type of lactic acid produced was the most constant character by which this species could be separated from *Lactobacillus casei*. Curran, Rogers, and Whittier (1933) suggested a close relationship between *Lactobacillus casei* and *Lactobacillus acidophilus*. Kulp and Rettger (1924) noted a relationship between *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*. However, Sherman and Hodge (1936) separated these two species rather readily, particularly by temperature of growth.

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Colonies of many species of the genus *Lactobacillus* are small, round, or lens-shaped and somewhat dense. In general, they would be considered as smooth types of colonies. Colonies of *Lactobacillus acidophilus* are usually distinguished by their filamentous, fine type of growth.

In studying strains of *Lactobacillus acidophilus* received from Cruickshank and the Kral Collection, it was observed that they formed smooth colonies and produced dextrorotatory lactic acid just as do cultures of *Lactobacillus casei*, contrary to the original description of this species. Many of the strains studied by Curran, Rogers, and Whittier and by others were then obtained for further study.

CULTURES STUDIED

As cultures were received, they were transferred to litmus milk, incubated at 32 C, and after sufficient growth occurred to acidify the milk, the transfers were stored at 1 C. Cultures were transferred at frequent intervals, sometimes being held as long as four months. In addition to milk, media ordinarily used contained 0.5 per cent tryptone, 0.3 per cent yeast extract, agar in solid media, and sugars in concentrations from 0.5 to 3 per cent.

The characters studied were morphology, colony type, growth in litmus milk, temperature of growth, fermentation of carbon compounds, and production of volatile and nonvolatile acids and carbon dioxide from sugar. Attempts were made by the selection of colonies and the use of phenol and lithium chloride in media to obtain the different colony types. Carbon dioxide production from glucose was observed in Eldredge tubes using barium hydroxide to absorb the carbon dioxide. Acid was titrated with N/10 sodium hydroxide, and carbon dioxide was determined by titration of excess barium hydroxide with N/10 sulfuric acid and calculation of the carbon dioxide absorbed. Lactic and volatile acids were determined by the method described by Pederson, Peterson, and Fred (1926).

The cultures studied included 14 strains isolated from dental caries, 8 from acidophilus milk, 10 from the human mouth, 2 from the intestinal contents of humans and 2 from rats, 2 from the human vagina, 4 from milk, 3 from cheese, 1 from butter, and several other strains identified as *Lactobacillus acidophilus*, as well as 9 authentic strains of closely related species including *Lactobacillus helveticus*, *Lactobacillus bulgaricus*, *Lactobacillus lactis*, and *Lactobacillus bulgaricus* var. *jugurt*. Besides the 34 cultures from various sources received from Curran, 3 from Cruickshank, and 3 from the Kral Collection, cultures from Orla-Jensen, Rettger, Sherwood, Sullivan, Demeter, Squibb Institute, Harrison, and the American Type Culture Collection were included in the series.

EXPERIMENTAL RESULTS

It was observed that although many of the cultures grew well in litmus milk when first obtained, reducing the litmus from the bottom and producing a solid curd, other cultures grew less readily and some would scarcely produce enough acid to curdle the milk. However, as the cultures were transferred several times, they became more active, growing readily in litmus milk or agar stabs. Milk was curdled rapidly and the litmus was reduced. Although no definite record was kept, it seemed that growth occurred more readily at 32 C than it did when the cultures were first received.

In broth the majority of strains were gram-positive short rods that often occurred in pairs or short chains. They stained rather poorly from milk with methylene blue. With few exceptions, cultures produced a smooth, round, or lens-shaped colony. Occasional colonies showed roughened edges. The type of colony could not be affected by the addition of lithium chloride or phenol to the media. Rough colony strains could be propagated from smooth strains only from culture no. 35. Culture no. 22 continued to produce rough strains throughout the study, and culture Sc changed from a rough to a smooth strain during the course of the study.

Most of the strains of related species could be distinguished from the *Lactobacillus casei* and *Lactobacillus acidophilus* strains by morphological as well as cultural characters. A few weak oral strains could be distinguished by several characters. The total acidity of the *Lactobacillus casei* and *Lactobacillus acidophilus* cultures that curdled milk varied from 1.23 to 2.13 per cent as lactic acid and the hydrogen ion concentration from pH 3.46 to 3.82.

In the fermentation of different sugars and related carbon compounds, the various cultures also showed a marked correlation. All cultures either failed to ferment or produced only a small amount of acid from glycerol, xylose, dextrin, and starch (figures 1a and 1b). A slightly greater activity was shown toward arabinose, rhamnose, and mannitol, and all or nearly all fermented fructose, glucose, mannose, galactose, sucrose, lactose, maltose, salicin, amygdalin, and α-methyl glucoside. Only in raffinose and inulin broths were variable results obtained (figure 1b) in that the majority of cultures failed to form acid but a few produced a marked acidity. These few exceptions could not be correlated with any of the other characters studied. Further, none of the cultures produced this higher acidity in both inulin and raffinose. Of the 13 cultures that produced high acidity in inulin, there were 3 cultures from dental caries, 1 from an anemia patient, 1 from an abnormal mouth, 3 from acidophilus milk, 1 from cheese, and 5 from milk.

Cultures of *Lactobacillus casei* showed an almost identical fermentation pattern except that no inulin fermenters were found (figure 2a and 2b).

The majority of the cultures grew at temperatures ranging from 18 to 45 C, the optimum varying from about 32 to 37 C. A few cultures failed to grow at 18 or 22 C, and several cultures grew exceptionally well at 45 C. With one exception, the latter group was of human origin, but the group does not contain all of such cultures. However, many of the related species of the genus, particularly those of more thermophilic nature, show a growth range different from that of the *Lactobacillus casei* and *Lactobacillus acidophilus* strains.

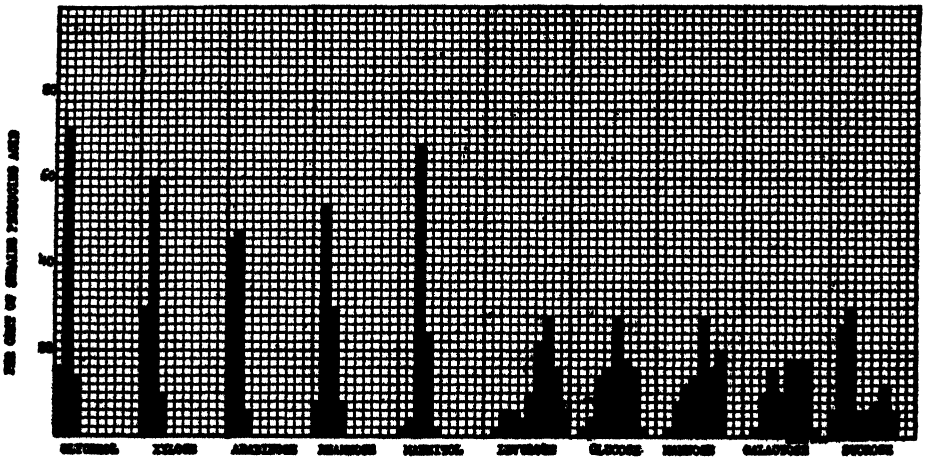

FIG. 1a

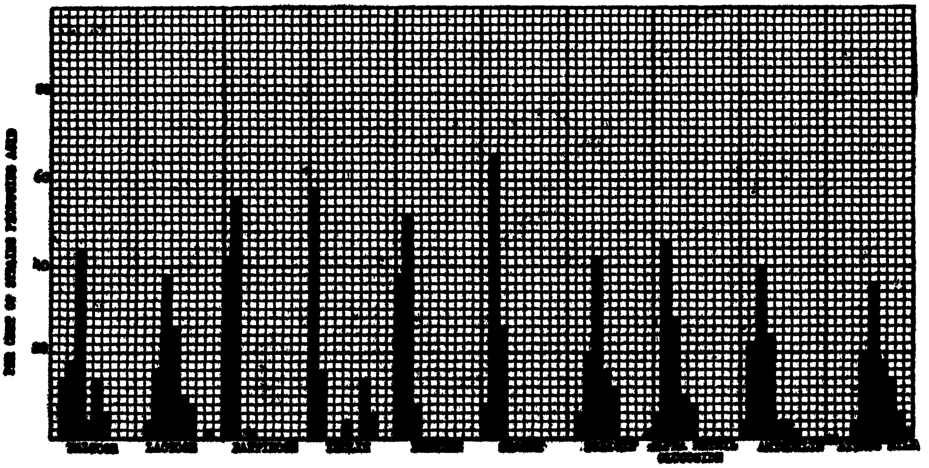

FIG. 1b

FIG. 1a AND b. FREQUENCY OF ACID PRODUCTION FROM VARIOUS CARBON COMPOUNDS BY CERTAIN CULTURES OBTAINED AS STRAINS OF LACTOBACILLUS ACIDOPHILUS EXPRESSED AS PER CENT OF STRAINS PRODUCING 0 TO 9 ML OF N/10 ACID IN 10 ML OF MEDIUM

Ten spaces used for each sugar signify, in the first block, percentage of strains producing no acid; in the second, percentage of strains producing 0.1 to 1 ml; in the third, 1.1 to 2.0, etc.

All cultures studied produced a small quantity of volatile acid (table 1) and a considerably greater quantity of lactic acid. With most strains of *Lactobacillus acidophilus* and *Lactobacillus casei*, the major part of this acid was dextrorotatory (table 1), but a few cultures produced some levorotatory acid.

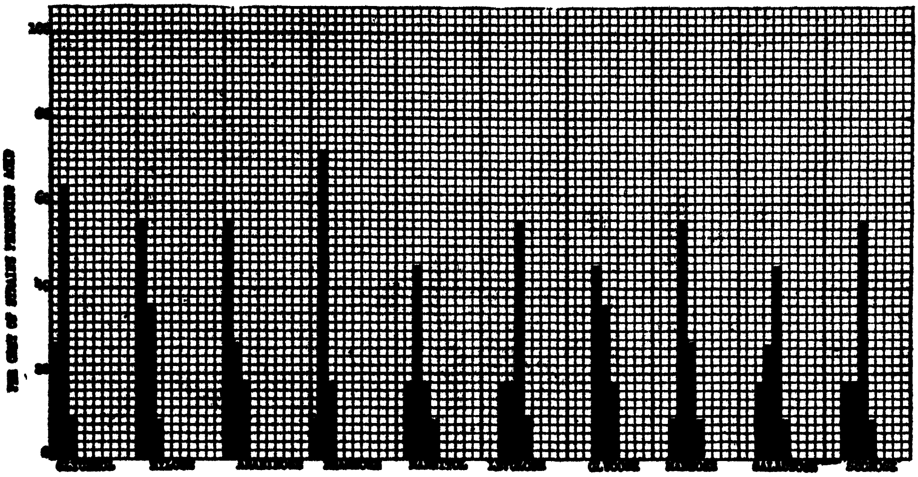

FIG. 2a

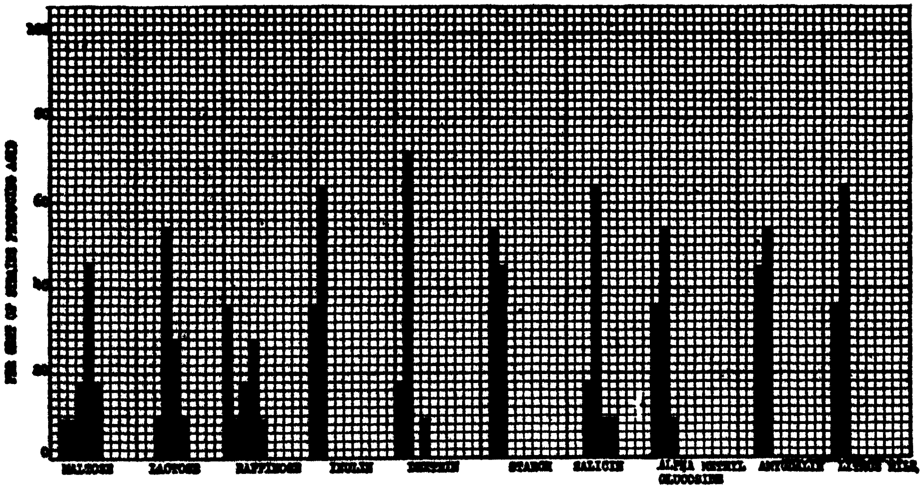

FIG. 2b

FIG. 2a AND b. FREQUENCY OF ACID PRODUCTION FROM VARIOUS CARBON COMPOUNDS BY CERTAIN STRAINS OF LACTOBACILLUS CASEI EXPRESSED AS PERCENTAGE OF STRAINS PRODUCING 0 TO 9 ML OF N/10 ACID IN 10 ML OF MEDIUM

Ten spaces used for each sugar signify, in the first block, percentage of strains producing no acid; in the second, percentage of strains producing 0.1 to 1 ml; in the third, 1.1 to 2.0, etc.

It was noticeable that very few of the cultures produced pure dextrorotatory acid and only a few cultures produced approximately equal quantities of the two forms. Culture no. 35 from acidophilus milk produced dextrorotatory

TABLE 1

Type of acid produced as an end product of fermentation by strains of the genus Lactobacillus

CULTURE NUMBER	VOLATILE ACID	NONVOLATILE ACID	ZINC LACTATE Water of crystallization		SPECIFIC ROTATION $[\alpha]^{20}_{D}$
			First fraction	Second fraction	
	grams	*grams*	*per cent*	*per cent*	
7469	.077	3.29	12.96	13.02	−8.1
L20	.119	2.47	13.49	13.78	−7.1
L20	.187	2.28	14.32		−5.2
L21	.081	2.12	13.88	13.14	−5.8
L23	.052	3.83	13.20	12.98	−6.4
L39	.155	5.66	13.64		
L0	.096	4.14	13.13	15.07	−6.4
L37	.089	2.27	12.96	12.94	−6.2
L37	.060	2.66	13.53	13.21	−7.5
L81	.056	3.71	13.45	13.87	−7.5
L14	.092	2.55	13.56	13.14	−7.1
L30	.114	2.31	13.05	13.86	−6.9
L30	.196	2.25	12.96	13.02	
58	.067	1.99	13.31		
65	.090	2.37	13.36	13.54	−7.4
79	.146	2.41	13.32		−7.1
82	.130	2.47	12.99		−7.4
90	.094	2.73	13.22		
92	.112	2.36	17.17		−2.3
95	.175	5.21	12.81		
99	.130	2.57	13.30		−7.8
22	.042	0.79	17.88	17.75	
45	.093	2.47	13.88	13.68	−6.8
31	.167	2.34	13.42	13.61	−7.4
39	.087	2.30	13.47	13.68	−7.5
40	.093	2.51	13.32	14.28	−7.5
33	.075	2.49	12.95		−7.9
44	.081	2.38	13.66	13.57	−7.1
35	.114	2.36	13.13		−7.8
35	.160	2.76	14.82		
35			17.22		
62	.098	2.49	13.17		−6.8
42	.106	2.39	13.18	13.26	−7.9
L38	.153	4.61	13.14		
Sc	.170	2.15	13.13		
Sc	.120	5.76	16.34		
RH24C			14.83		
RH5E			18.08		
EH22G			18.04		
S42A	.337	5.60	13.14		
S137	.166	1.63	17.99		
S10	.151	5.71	13.20		
S12	.239	5.48	12.91		
L56	.236	4.98	13.13		
S34	.350	5.62	12.11		

TABLE 1—*Concluded*

CULTURE NUMBER	VOLATILE ACID	NONVOLATILE ACID	ZINC LACTATE		SPECIFIC ROTATION $[\alpha]_D^{20}$
			Water of crystallization		
			First fraction	Second fraction	
	grams	*grams*	*per cent*	*per cent*	
L75	.173	5.50	18.07		
L5	.111	4.44	18.04		
MNH	.270	5.91	13.02		
S23	.050	1.60	13.58		
Wick	.220	6.48	12.99		
S5	.240	6.41	18.12		
L13	.157	2.45	17.89		
L31	.087	2.07	15.50		+2.7
L43	.072	4.17	18.09		
L33	.090	2.31	13.25		+7.2
Jugurt 13	.082	2.07	18.14		
7993	.325	5.24	13.08		
7995	.215	4.68	13.01		
8001	.203	3.29	14.95		
L80	.075	4.28	18.13		

acid in the first determination. However, rough strain selections showed greater production of levorotatory acid, the water of crystallization of the zinc salts changing from 13.13 to 14.82, and then to 17.22 per cent in three subsequent fermentations. The theoretical amount for pure active acid is 12.97, and that for inactive or a mixture of dextrorotatory and levorotatory acid is 18.17 per cent. Likewise, when culture Sc was first studied, the lactic acid produced contained approximately ⅓ levo- and ⅔ dextrorotatory acid, but later almost pure dextrorotatory acid was formed.

GROUPING OF STRAINS AND CORRELATION OF CHARACTERS

A large majority of the cultures studied produced a fairly high amount of acid in litmus milk accompanied by curdling and a definite reduction of litmus. They stained faintly with methylene blue and very often cells resembled ghost cells with small granules which have somewhat the appearance of cocci. Often there was a clear zone around the cell. They grew poorly in milk at 18 C, rapidly at 37 to 40 C, but with less acid production than at 32 to 37 C. Among this group the only marked differences in ability to ferment carbon compounds was noted in the ability of some strains to ferment inulin and a few to ferment raffinose. Variation in the amount of acid produced from sucrose was observed.

A smaller but closely related group was similar in its fermentations but always produced less acid from sugar and a softer curd in milk.

An attempt was made to separate cultures into groups on the basis of difference in characters, particularly fermentation characters. However, when such a separation was made, no correlation could be noted. For example, the 13 inulin-fermenting strains had no other character in common to distinguish them from the group as a whole. The same was true of other fermentations as well as temperatures of growth, reduction of litmus, or other characters.

Nearly all of the strains studied had either been shown by previous workers or in the present study to produce dextrorotatory lactic acid in excess over levorotatory lactic acid. Those strains, with few exceptions, which had previously been found to produce inactive lactic acid by Curran, Rogers, and Whittier, and also to produce rough colonies, were found in this study to produce dextrorotatory lactic acid and smooth colonies. Cultures 92, RH5E, EH22G, and 22 continued to form inactive acid, but the first three produced a high acidity in sugars and formed smooth colonies.

A smaller group of organisms (cultures S137 and S5) are unlike the group as a whole and are not similar to the rough strain of *L. acidophilus* (e.g., culture no. 22) and may possibly be related to the types described by Thjötta, Hartmann, and Boe (1939), Robin (1847), and Kligler (1915). A few cultures (L75, L5, and L13) are more like *Lactobacillus plantarum* than they are like *L. casei* or *L. acidophilus*.

Typical cultures of *Lactobacillus bulgaricus* (cultures 7995 and 8001), *Lactobacillus lactis* (culture L33), *Lactobacillus helveticus* (culture L31, L43, L80, and 7999), and *Lactobacillus bulgaricus* var. *jugurti* (cultures J13 and 7993) were readily distinguished, although it may be noted that cultures originally designated as strains of one or the other of these species are included in the larger grouping above.

DISCUSSION

The results indicate that some of the cultures studied have changed their characters during a period of years. These changes imply that the species *Lactobacillus acidophilus* (Moro) Holland is closely related to *Lactobacillus casei* (Orla-Jensen) Holland, the differences centering around their type of growth. When some of these cultures were studied by Curran, Rogers, and Whittier, they were found to form rough or filamentous colonies with only a few smooth colonies. They further formed inactive lactic acid with volatile acid, usually did not grow at 20 C, curdled milk slowly, rarely fermented mannitol, and usually fermented raffinose. Similar characters were found by others who studied the acidophilus type organisms included in the study. However, during the intervening time, in which it was observed that cultures grew better in milk, the characters apparently have changed. In the present studies the characters found were similar to those of the typical cultures of *Lactobacillus casei* in that they now form smooth colonies on agar, curdle milk with a firm curd producing comparable amounts of acid, ferment the same sugars, and produce dextrorotatory lactic acid.

It is possible that Orla-Jensen's observations in regard to the changes in character of his species *Streptobacterium casei* (*Lactobacillus casei*) may have been of the same nature. Orla-Jensen noted a change in type of acid formed as well as changes in the fermentation of sugars. He did not report the colony type. There is little doubt that the changes from rough to smooth colonies and from inactive lactic acid to dextrorotatory acid, as observed by Kopeloff and Kopeloff (1937), were the same as herein reported. Kopeloff (1934) was unable to cause the smooth forms to revert to the rough form. In spite of repeated attempts, the best that could be done in the present studies was to obtain filamentous edges on a few colonies and a reversion to inactive lactic acid in one instance.

SUMMARY

A comparative study of a number of cultures of lactobacilli from milk products, dental caries, the intestine, and similar sources has shown that these strains have many characters in common. The cultures isolated from milk and milk products usually considered as strains of *Lactobacillus casei* (Orla-Jensen) Holland form smooth colonies and dextrorotatory lactic acid. A number of cultures isolated as rough or filamentous colonies from various parts of the body, and usually considered as strains of *Lactobacillus acidophilus* (Moro) Holland, have changed their characters over a period of years and are now identical with cultures of *Lactobacillus casei*. It is concluded that *Lactobacillus acidophilus* and *Lactobacillus casei* are rough and smooth strains of a single type.

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THE GROWTH OF CLOSTRIDIUM SEPTICUM AND ITS INHIBITION

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Chemical relatives of essential metabolites have been found to be agents for the inhibition of the growth of bacteria, and some of these have been used in the therapy of bacterial infections (Welch, 1945; Roblin, 1946; Woolley, 1946*a*). In the course of studies on the chemotherapy of gas gangrene we have investigated the nutritional requirements of *Clostridium septicum* with the intention of testing the effect of chemical relatives of growth factors upon its growth. Bernheimer (1944) has shown that a strain of this organism requires, among other things, pantothenic acid for growth. We (Ryan *et al.*, 1945) have shown that this requirement is due to an inability to synthesize the pantoyl moiety of the pantothenate molecule. The other portion of the molecule, β-alanine, is normally synthesized and coupled with the added pantoyl moiety to form pantothenate. The present paper reports studies on the effect of chemical relatives of these precursors and of pantothenate upon the *in vitro* growth of two strains of *C. septicum*, one of which is able to synthesize all parts of the pantothenate molecule.

EXPERIMENTAL

The chemically defined basal medium which was finally selected for the growth of *C. septicum* is a modification of that proposed by Bernheimer, and directions for its composition are as follows:

To 500 ml of distilled water add:
10 g glucose
20 g casamino acids
2.65 g monopotassium phosphate
7.14 g disodium phosphate
75 mg calcium chloride

Treat the solution for 30 minutes at room temperature with 5 g of norite A and filter through hyflo.

Add:
20 mg *l*(−)tryptophane, dissolved in a small amount of water with heat
150 mg *l*(−)cystine, dissolved in a small amount of water with hydrochloric acid and heat
20 mg glutamine
800 mg potassium bicarbonate
1 μg biotin, crystalline-free acid
1 mg thiamine hydrochloride

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[1] This work was supported by a grant from the Josiah Macy, Jr., Foundation.

1 mg nicotinic acid
1 mg pyridoxine (pyridoxal or pyridoxamine can be substituted in an equal quantity without affecting the growth of *C. septicum*)
5 ml ferric sulfate solution made by dissolving 357 mg in a liter of distilled water with hydrochloric acid
2 ml salt solution, made by dissolving 50 mg $Cu\ SO_4 \cdot 5\ H_2O$, 50 mg $Zn\ SO_4 \cdot 7\ H_2O$, 20 mg $Mn\ Cl_2 \cdot 4\ H_2O$, 22.5 mg $Mg\ SO_4 \cdot 7\ H_2O$, and 1 ml of concentrated hydrochloric acid in 100 ml of distilled water

Bring to one liter and adjust the pH to between 7.0 and 7.5 with sodium hydroxide.

This medium may be stored under toluene in a refrigerator for at least 5 days. We have routinely prepared fresh medium every 2 or 3 days. Before use the proper amount of neutralized cysteine hydrochloride to make a 0.2 per cent solution was added. The cysteine was neutralized to phenol red in a small amount of water with 1 molar sodium hydroxide. The solution was added immediately to the culture medium, which was then sterilized by autoclaving for 10 minutes at 15 pounds' pressure. Under optimal conditions at 37 C, growth on this basal medium was complete in about 18 hours. In order to obtain the size of the final crop of bacteria, culture tubes were routinely measured after about 40 hours.

The yield of bacteria was determined routinely by the use of a densitometer. When necessary, cultures were diluted with 0.9 per cent sodium chloride to bring the density within the range of the instrument. A micro-Kjeldahl determination of bacterial nitrogen was made on washed cultures of strain 59 Li of *C. septicum* which had grown in complete medium for 20 hours. The densitometer was then calibrated in terms of mg of nitrogen found in such cultures, and readings have been expressed as mg N per 100 ml of culture. The average deviation of this method was about 5 per cent.

The number of viable organisms per ml in 40-hour cultures of strain 59 Li, determined by colony counts on blood agar plates, was 2×10^7 organisms, equivalent to 0.15 mg of bacterial N. Direct hemocytometer counts confirm this equivalence. However, during the early part of the logarithmic phase of growth, direct microscopic observation showed that about one-quarter of the organisms were in the form of the long unsegmented cells frequently found among bacteria whose growth is incompletely inhibited. The presence of these long cells required a very thorough shaking of the first dilution flask in the plating procedure in order that fragmentation would not cause the appearance of greater numbers of colonies as the dilutions progressed.

Consistent colony counts were obtained on both blood agar and complete medium plates when these were inverted over alkaline pyrogallol in Bray dishes. However, in plates made up with our basal medium and washed agar, results under similar conditions were very inconsistent. The same was true when the plates were incubated in desiccators which had been evacuated and refilled with gases such as nitrogen and hydrogen, with or without added carbon dioxide (Rockwell, 1921). Irregular results were also obtained when the plates, after cooling, were filled to the brim with fresh agar medium containing 0.2 per cent cysteine but no cells. Apparently on the chemically defined medium the gaseous requirements of strain 59 Li are rather precise.

Success in making colony counts on the basal medium was attained by the use of the semisolid agar procedure developed by Redowitz (1941) for cultures of *Lactobacillus acidophilus*. This involved the dilution of a culture of *C. septicum* with basal medium containing cysteine. One ml of the appropriate dilution was added to 9 ml of basal medium containing cysteine and 0.15 per cent washed agar. This had been melted and kept at 44 C in a test tube. This mixture was taken up and ejected from a 1-ml blow-out pipette 10 times, and then 1 ml was transferred with the same pipette to the next tube of semisolid agar. From the last tube 1 ml of medium was discarded after mixing, and in this way tenfold dilutions were obtained. The tubes were incubated at 37 C for about 24 hours, when they showed cottony but discrete colonies. About 30 colonies is the maximum number that can be conveniently counted in 9 ml of semisolid medium about 3 inches deep. Determinations by this method of the number of bacteria in nine samples from a single culture of *C. septicum* gave an average value of $3.7 \pm 0.4 \ (\sigma_M) \times 10^7$ per ml.

Stock cultures of the bacteria were grown on an egg-meat medium at 37 C and then stored (presumably as spores) at room temperature. Inocula were prepared by transferring a loopful of the egg-meat medium to test tubes containing about 15 ml of the complete medium. This medium consisted of 0.25 per cent sodium chloride, 0.1 per cent sodium bicarbonate, 0.34 per cent disodium phosphate, 0.082 per cent monopotassium phosphate, 0.5 per cent glucose, 2 per cent Difco tryptose, 0.3 per cent Difco yeast extract, and 0.1 per cent neutralized cysteine hydrochloride; the pH was 7.4. Growth in this medium was allowed to take place between 10 and 18 hours, at which time the experimental tubes were inoculated with a loopful of bacterial suspension. The data in the tables and figures of this paper are averages of at least duplicate determinations.

Two strains of *C. septicum*, 59 Li and 44, were obtained from Dr. Alan Bernheimer, to whom we are indebted. Early in our studies of strain 59 Li it was found that under standard conditions the length of the lag period varied considerably on our basal medium. Indeed, frequently among a series of mated tubes no growth appeared in some. It was found that this behavior could be completely eliminated by incorporating the pyrimidine, uracil, in the medium. When this was done, growth was regularly complete in 18 hours, and growth failures were not encountered. A culture of 59 Li which had grown up in the absence of uracil was plated out, and strain 59 Li A was isolated. This new strain showed consistent growth that was independent of uracil. The genetics of this situation is the subject of a separate communication (Ryan *et al.*, 1946). Strain 59 Li A was used instead of its parent in most of the following experiments.

RESULTS

Nutritional requirements. We have confirmed the observation of Bernheimer (1944) that the vitamins, pyridoxine, nicotinic acid, thiamine, and biotin, are required for the growth of strain 59 Li of *C. septicum*. In addition, strains 59

Li and 59 Li A, but not strain 44, require *d*-pantothenic acid (3 μg per ml). The latter requirement can be satisfied by the addition of sodium *dl*-pantoate or pantoyl lactone to the medium (Ryan *et al.*, 1945). Our basal medium, however, differs from that of Bernheimer in several respects. The carbon treatment and filtration remove not only a slight precipitate but also such contaminants of the casamino acids as pantothenate, pantoate, or pantoyl lactone. Strain 59 Li, which requires pantothenic acid or the pantoyl moiety, showed no growth whatsoever on our basal medium until one of these compounds was added. Bernheimer, on the other hand, showed that in the absence of pantothenic acid strain 59 Li grew on his medium to about 35 per cent of its maximum yield. In the following experiments with 59 Li A, which has the same pantothenate requirements as its parent strain, 59 Li, calcium *d*-pantothenate was present in the medium in a concentration of about 3 μg per ml. Unless otherwise specified strain 44 was grown in medium without added pantothenate.

In our hands cysteine hydrochloride proved a better reducing agent for bacterial growth than thioglycolic acid. A concentration between 0.1 and 0.2 per cent was optimum.

Autoclaving the basal medium was found to be much simpler than making the series of sterile additions proposed by Bernheimer. Such treatment resulted in a growth of strain 59 Li A (14.4 mg N per 100 ml) equal to that obtained on Bernheimer's medium (with 0.2 per cent cysteine, 13.4 mg N per 100 ml). Nevertheless when our basal medium to which cysteine had been added was not autoclaved but was sterilized by filtration through a sintered glass bacterial filter crucible, growth was increased to 19.8 mg N.

This difference is not associated with the toluene used for preservation of the basal medium which is boiled off during autoclaving. The stimulation appeared after sterile filtration of fresh medium, to which toluene had never been added, as well as after filtration of medium which had been preserved under toluene for 2 days. The increased crop of bacteria on the sterile filtered medium was also not due to the removal of some toxic factor during filtration, because we failed to find such stimulation when autoclaving followed or preceded sterile filtration. On the other hand, the time of autoclaving bears a definite relation to the yield of bacteria. In an experiment in which sterile-filtered medium after 48 hours supported growth equivalent to 17.1 mg N per 100 ml, autoclaving for 20, 10, and 5 minutes resulted in final growths of 15.0, 16.2, and 17.7 mg N per 100 ml, respectively. This, and the fact that a slight insoluble precipitate is formed upon autoclaving, suggests that the effect of autoclaving might be due to the loss of some factor in the medium. Glutamine, for example, would be expected to be almost completely destroyed upon autoclaving. However, when glutamine was sterile-filtered separately and added to autoclaved medium, no increase in growth after 48 hours was noted (15.9 and 16.5 mg N per 100 ml). Moreover, the hypothesis of loss by autoclaving does not seem tenable in view of the fact that the final crop of bacteria did not increase in media where the concentration of vitamins, casamino acids, sugar, or salts was doubled or halved. The concentration of these substances in the whole medium was optimum for the growth of strain 59 Li A of *C. septicum*.

It appears, rather, that the effect of autoclaving may be due to the production of some toxic substance(s) from material in the medium. It is known that glucose, when autoclaved under alkaline conditions, will form many different compounds (Evans, 1929). Table 1 lists the yields of strain 59 Li A obtained when glucose was sterilized separately and then added to the medium. The medium was made up as previously mentioned except that the glucose and phosphate were omitted at first. In the case of the autoclaved and sterile-filtered controls the glucose and phosphate which were not carbon-treated were added to the medium just before it was sterilized. The experiments consisted of the sterile additions of the specified substances to the remainder of the medium, which was autoclaved with or without phosphate. Autoclaving was at 15 pounds for 10 minutes, and sterile filtration was through a sintered glass bacterial filter. The controls yielded somewhat less growth than when our medium was prepared as usual, but sterile filtration was definitely better than autoclaving. Likewise, whenever glucose was sterile-filtered, growth was increased slightly. However, when the glucose was autoclaved separately and then added to the medium, there was a considerable increase in growth. Apparently autoclaving glucose separately or with phosphate produced some stimulating factor, which was not pyruvic acid (Smiley, Niven, and Sherman, 1943), but when glucose was autoclaved with the rest of the medium, inhibiting factors were produced as well. Our analysis of this interaction is admittedly incomplete, and certainly more substances than glucose are involved. Nevertheless, we interpret the effect of sterile filtration as due to the absence of formation of inhibitory materials, whereas the limit of growth on autoclaved medium is balanced by both inhibitory and stimulatory factors, which are formed during autoclaving. Strain 44, on the other hand, does not show a difference in the amount of growth in sterile-filtered as compared with autoclaved medium.

TABLE 1

Growth of strain 59 Li A after 48 hours in media to which glucose was added after being separately sterilized

TREATMENT OF GLUCOSE	MG N PER 100 ML
Autoclaved in medium	10.8
Sterile-filtered with medium	16.1
Autoclaved separately	15.3
Sterile-filtered separately	11.4
Autoclaved with phosphate	15.3
Sterile-filtered with phosphate	12.8

During the growth of strain 59 Li A the pH of the basal medium fell from neutrality to 5.1. Cessation of growth was not due to the acidity of the medium alone, for partially grown cultures when washed and transferred to fresh medium of pH 5.1 continued their growth. Moreover, increasing the buffer capacity of the medium by doubling the salt concentration did not increase the yield of bacteria. However, the optimum pH is between 7.0 and 7.5 when growth equivalent to 16.6 and 17.1 mg N per 100 ml was obtained after 48 hours. At pH's of 6.5 and 8.0 somewhat less growth, or 14.4 and 14.1 mg N per 100 ml, resulted.

The factor(s) limiting final growth can be modified by the addition of natural products such as Difco tryptose or Fleischman's yeast extract, as table 2 shows. Yeast extract seems more active for strain 59 Li than tryptose, but strain 44 may be more sensitive to the inhibitors it contains. Although the 2-day yields shown in table 2 were influenced by tryptose, the rate of growth was not (figure 1).

TABLE 2

Yield of bacteria in mg N per 100 ml of medium plus tryptose or yeast extract after 48 hours

STRAIN CONCENTRATION MG PER ML	59 LI A		44	
	Tryptose	Yeast extract	Tryptose	Yeast extract
0	14.8	15.7	8.5	8.5
1	14.7	16.8	9.0	10.1
3	—	19.4	—	—
10	18.2	17.7	16.7	12.0
20	21.9	18.4	19.3	13.8
40	13.9	—	17.2	—
100	15.6	3.0	15.3	5.7

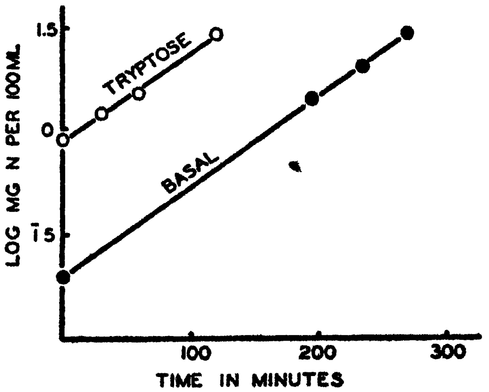

FIG. 1. THE RATE OF GROWTH OF STRAIN 59 LI A IN THE PRESENCE (OPEN CIRCLES) AND ABSENCE (SOLID CIRCLES) OF 20 MG TRYPTOSE PER ML

The time has been designated from an arbitrary zero point since the lag period in tryptose is much shorter than in its absence.

In 14 determinations of the generation time of strain 59 Li A during the logarithmic phase of growth on medium supplemented with 20 mg tryptose per ml, an average of 70 minutes was obtained, whereas 62 minutes was the average generation time of the controls. Tryptose does, however, influence the length of the lag period. The time required to reach a growth equivalent to 2 mg N per 100 ml averaged about 1,100 minutes in three experiments on our basal medium, but the presence of 20 mg of tryptose per ml shortened this time to about 500 minutes.

A wide variety of growth factors were tested in an attempt to duplicate the stimulation caused by tryptose. Among these were tested, individually and in combination, 19 amino acids, asparagine, guanine, adenine, xanthine, uracil, yeast nucleic acid, choline, glutathione, pyruvic acid, oleic acid, folic acid concentrate, vitamin B_c and B_c conjugate, pimelic acid, *p*-aminobenzoic acid, inositol, riboflavin, pyridoxal, pyridoxamine, welchii factors (Ballentine *et al.*, 1944), and iron.[3] None of these substances increased the yield of bacteria on our medium.

It is possible that some polypeptide growth factor, like strepogenin (Sprince and Woolley, 1945; Womack and Rose, 1946) is present in tryptose and responsible for its effect on *C. septicum.* The addition of 1 mg of Difco casamino acids per ml did not reproduce the tryptose stimulation. It is also possible that tryptose may bind some trace element present in toxic quantities (Hutner, 1946), although this seems improbable in view of the absence of effect when the salt composition of the medium was doubled or halved. Tryptose contains material(s) active in supporting the growth of uracil-requiring strains of *Neurospora crassa*, *Escherichia coli*, and *Shigella paradysenteriae.* This material cannot, however, be responsible for the effect of tryptose on the uracil-independent strain of *C. septicum*, 59 Li A. The effect of tryptose may be due to its content of several factors influencing the lag period and the final growth independently. The growth of strain 44 on basal medium is irregular in the sense that an appreciable fraction of a series of similar cultures fail to grow. Bernheimer (personal communication) suggests that carbon dioxide or bicarbonate is important for the initiation of growth of strain 44. In our autoclaved medium the bicarbonate would be lost. However, the incorporation of 20 mg of tryptose per ml, but not of purines or pyrimidines, results in the growth of all cultures. It may be that there is some substance in tryptose required for the growth of strain 44, and that growth in its absence is due to mutation to independence of this factor just as in the case of the uracil requirement of strain 59 Li (Ryan *et al.*, 1946). Moreover the absence of glutamine from the basal medium results in a similarly erratic growth of strain 59 Li. Glutamine itself is destroyed under the conditions of pH and temperature used in sterilization, whereas glutamic acid is a component of the basal medium. The effect of glutamine may be due to the existence of organisms independent of some contaminating heat-stable factor (Ballentine *et al.*, 1947). Neither the genetic nor the chemical nature of these effects has been investigated.

One of the vitamin analogues, sodium β-pyridine sulfonate, which was studied as a possible inhibitor of growth (McIlwain, 1940) actually increased the bacterial crop. The average 48-hour yield of five experiments with 59 Li A in which 10 mg of this compound per ml were present was 17.1 mg per 100 ml, compared to the control yield of 12.8 mg N per ml. The presence of 1 mg per ml resulted in a similar increase. Raoul (1945) found that high concentrations of 3-pyridine sulfonic acid inhibited the growth of *E. coli* and *Bacillus proteus*, although lower concentrations favored growth. Since the addition of nicotinic acid in concentrations from 0.25 to 4.0 µg per ml did not result in increased growth, it is improbable that the stimulatory action of β-pyridine sulfonate is due to its substitution for the analogous vitamin, nicotinic acid. The action of sodium β-pyridine sulfonate is probably different from that of tryptose or of sterile filtration, for when combined with these treatments the effects seem to be additive. The same is true of the action of tryptose and sterile filtration (table 3). A combination of the last two yielded the maximum growth of strain 59 Li A of *C. septicum* that we have obtained—24 mg of bacterial nitrogen per 100 ml of culture medium.

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[3] The authors wish to thank the following for supplying them with certain growth factors; Dr. R. J. Williams for the folic acid concentrate, Dr. J. Pfiffner for the vitamin B_c conjugate, and Dr. E. E. Snell for the pyridoxal and pyridoxamine.

The incomplete nature of our basal medium is also apparent in another way. There are a minimum number of cells of *C. septicum* (between 10^2 and 10^3) which when inoculated into 10 ml of liquid basal medium will result in growth. Since the inocula were centrifuged and resuspended in basal medium three times, it is not likely that the effect of inoculum size is due to a carry-over of some required material from the complete medium. In 10 ml of basal medium rigidified with 0.15 per cent semisolid agar, single colonies are frequently found in tubes containing 10^{-7} dilutions. Thus, it is possible that the cells are slowly able to synthesize some required catalytic substance which can be lost in the medium. In very dilute bacterial suspensions this material is lost too rapidly for growth to take place. On the other hand, in semisolid agar medium the material may not be carried away rapidly enough to bring the concentration in the environment of the cell to a subthreshold level. It is also possible that the relationship between growth and inoculum size is not due to a stimulatory substance but rather to an inhibitor, which seems to be the case in the tubercle bacillus (Davis and Dubos, 1946).

TABLE 3

Growth of strain 59 Li A in mg N per 100 ml after 48 hours in basal medium to which combinations of supplements had been added and which was sterilized by different methods

METHOD OF STERILIZATION	SUPPLEMENT	MG N PER 100 ML
Autoclaved	None	13.7
Autoclaved	20 mg tryptose per ml	18.8
Autoclaved	10 mg sodium-β-pyridine sulfonate per ml	16.8
Autoclaved	Tryptose and sodium-β-pyridine sulfonate	19.6
Filtered	None	17.1
Filtered	20 mg tryptose per ml	24.0
Filtered	10 mg sodium-β-pyridine sulfonate per ml	18.8

Inhibitors. Besides studies on the chemical analogues of pantothenic acid and its precursors, a few experiments with derivatives of other vitamins were performed. In concentrations from 1 to 10 mg per ml, γ-(3,4-ureylenecyclohexyl)-

butyric acid[3] and desthiobiotin did not appreciably decrease the growth of strain 59 Li A and were not studied further.

Sodium *dl*-pantoyltaurine, the sulfonic acid derivative of pantothenic acid, and sodium γ-hydroxy buteryl taurine,[4] a compound in which both parts of the pantothenate molecule have been modified, were chosen as possible antagonists

TABLE 4

The effect of chemical relatives of pantothenic acid and its precursors on the growth of the pantothenate-independent strain 44 expressed as percentage of the 48-hour bacterial crop produced by controls grown in the absence of these substances

ANALOGUE 10 MG PER ML	NONE	SUPPLEMENTS TO BASAL MEDIUM	
		0 35 µg Sodium *dl*-pantoate per ml	0.01 µg Calcium *d*-pantothenate per ml
Sodium *dl*-pantoyltaurine	90	102	81
Sodium γ-hydroxy buteryl taurine	—	83	92
Sodium γ-hydroxy buterate	0	0	21
dl-Pantoic amide	93	122	113
dl-β-Amino butyric acid	—	—	88
Taurine	100	107	92

TABLE 5

The effect of chemical relatives of pantothenic acid and its precursors on the 48-hour crop of bacteria produced by the pantothenate-dependent strain 59 Li A

ANALOGUE 10 MG PER ML	PER CENT OF 14.4 MG N PER 100 ML*	SUPPLEMENTS TO BASAL MEDIUM			
		0.35 µg Sodium *dl*-pantoate per ml		0.01 µg Calcium *d*-pantothenate per ml	
		Per cent of controls	Per cent of 14.4 mg N per 100 ml*	Per cent of controls	Per cent of 14.4 mg N per 100 ml*
Sodium *dl*-pantoyltaurine	73	758	79	619	85
Sodium γ-hydroxy buteryl taurine	—	116	19	107	67
Sodium γ-hydroxy buterate	27	400	35	244	26
dl-Pantoic amide	98	1034	88	757	104
dl-β-Aminobutyric acid	—	—	—	111	14
Taurine	0	34	5	24	2
None—control	0	100	11	100	19

* 14.4 mg N per 100 ml was the amount of growth produced on an optimum amount (3 µg per ml) of calcium *d*-pantothenate in the absence of the pantothenic analogues.

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[3] In low concentrations this compound is known to inhibit the growth of yeast, *Lactobacillus casei* (English *et al.*, 1945) and *Lactobacillus arabinosus* (Axelrod *et al.*, 1946). We wish to thank Dr. J. O. Lampen for supplying us with a sample.

[4] Sodium γ-hydroxy buteryl taurine, not previously described, was prepared by the fusion for 3 hours at 125 C of γ-hydroxy buteryl lactone and sodium taurine in equimolar quantities. The substance was recrystallized from isopropyl alcohol and ether.

of pantothenic acid. Taurine and *dl*-β-aminobutyric acid were selected as analogues of β-alanine, and sodium γ-hydroxy buterate and *dl*-pantoic amide as relatives of pantoyl lactone. Table 4 shows the effect of these compounds on the growth of strain 44 of *C. septicum*, which is independent of an external supply of pantothenic acid. The only compound with an appreciable effect in the relatively high concentration of 10 mg per ml was sodium γ-hydroxy buterate. The growth of the pantothenate-requiring strain, 59 Li A, on the other hand, is inhibited by taurine but not by the other pantothenate analogues (table 5). Indeed, some of the other pantothenate analogues actually supported the growth of 59 Li A in the absence of pantoate and pantothenate, as shown in the first column of table 5. These compounds, sodium γ-hydroxy buterate, *dl*-pantoic amide, and sodium *dl*-pantoyltaurine, also stimulated growth in the presence of suboptimal amounts of pantoate and pantothenate. The percentage to which this stimulation approximated maximum growth is indicated in the third and fifth columns of table 5.

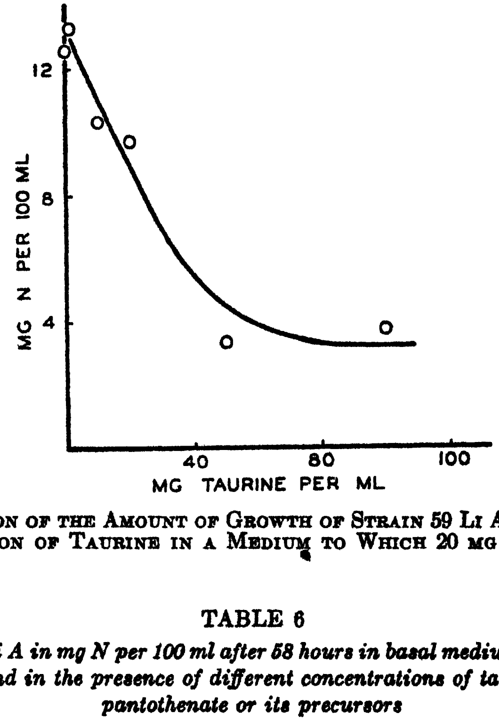

FIG. 2. THE RELATION OF THE AMOUNT OF GROWTH OF STRAIN 59 LI A AFTER 58 HOURS TO THE CONCENTRATION OF TAURINE IN A MEDIUM TO WHICH 20 MG TRYPTOSE PER ML HAS BEEN ADDED

TABLE 6

Growth of strain 59 Li A in mg N per 100 ml after 58 hours in basal medium plus 20 mg tryptose per ml and in the presence of different concentrations of taurine and pantothenate or its precursors

TAURINE MG PER ML	CALCIUM *d*-PANTOTHENATE µg PER ML			SODIUM *dl*-PANTOATE µg PER ML		β-ALANINE µg PER ML
	3	100	1,000	100	1,000	1,000
0	12.6	11.0	9.1	10.6	5.9	7.6
50	3.3	2.8	4.8	3.3	2.6	4.7

Complete inhibition of the growth of strain 59 Li A is not achieved with concentrations of taurine even approximating 1 molar in strength (figure 2). Table 6 shows that there may be a small reversal of taurine inhibition by increasing the pantothenate concentration or by the addition of β-alanine. If real, this

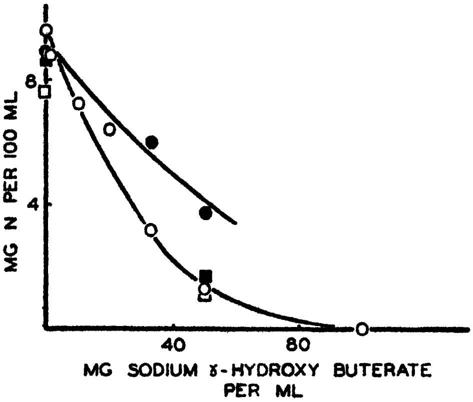

FIG. 3. THE INHIBITION OF THE GROWTH OF STRAIN 44 BY DIFFERENT CONCENTRATIONS OF SODIUM γ-HYDROXY BUTERATE AFTER 58 HOURS IN A MEDIUM CONTAINING 20 MG TRYPTOSE PER ML AND EITHER 1 MG CALCIUM *d*-PANTOTHENATE PER ML (SOLID CIRCLES), 1 MG SODIUM *dl*-PANTOATE PER ML (OPEN SQUARES), 1 MG β-ALANINE PER ML (SOLID SQUARES), OR NO ADDED SUPPLEMENT (OPEN CIRCLES)

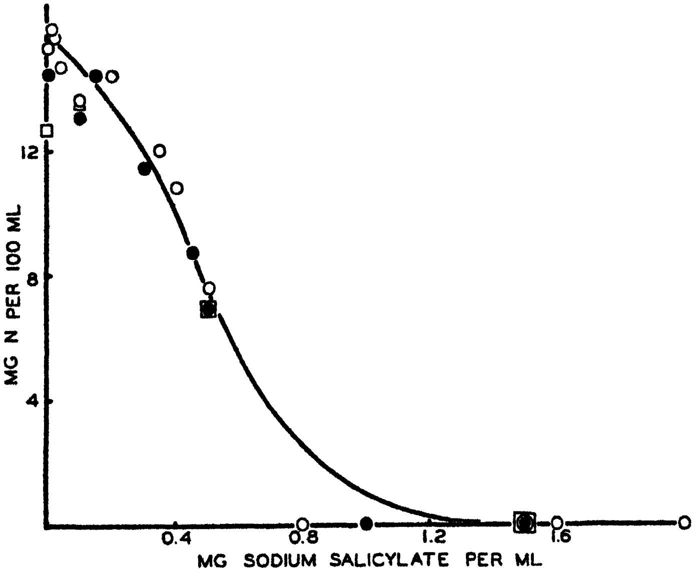

FIG. 4. THE INHIBITION OF THE GROWTH OF STRAIN 59 LI A BY DIFFERENT CONCENTRATIONS OF SODIUM SALICYLATE AFTER 48 HOURS IN A MEDIUM DEVOID OF TRYPTOSE BUT CONTAINING EITHER 3.2 μg CALCIUM *d*-PANTOTHENATE PER ML (OPEN CIRCLES), 100 μg CALCIUM *d*-PANTOTHENATE PER ML (CLOSED CIRCLES), OR 100 μg SODIUM *dl*-PANTOATE PER ML (OPEN SQUARES)

effect is slight and is not brought about by the addition of pantoate. The fact that strain 59 Li A grows at all in the absence of added pantoate or pantothenate but in the presence of β-alanine is due to the activity of tryptose in the medium, which in independent assays seemed to contain the equivalent of about 0.28 µg of pantothenate per mg.

The growth of strain 44 can be completely inhibited by a concentration of approximately 1 molar sodium γ-hydroxy buterate (figure 3). Moreover, the inhibition can be at least partially reversed by the presence of high concentrations of pantothenate. Sodium *dl*-pantoate and β-alanine, in the concentrations tried, had no effect in reversing the inhibition. Apparently an antagonist of sodium γ-hydroxy buterate inhibition is to be found in tryptose, as a comparison of the degree of inhibition by 10 mg of inhibitor per ml in a medium with (figure 3) and without tryptose (table 4) will show. The same seems to be true of taurine inhibition of strain 59 Li A (figure 2 and table 5).

TABLE 7

Growth of strains 59 Li A and 44 in mg N per 100 ml after 58 hours in the basal medium plus 20 mg tryptose per ml and in the presence of different concentrations of salicylate and pantothenate or its precursors

SODIUM SALICYLATE MG PER ML	CALCIUM *d*-PANTOTHENATE µg PER ML			SODIUM *dl*-PANTOATE µg PER ML		β-ALANINE µg PER ML
	3	100	1,000	100	1,000	1,000
			Strain 59 Li A			
0	15.2	14.5	12.4	13.7	11.1	11.7
0.5	4.9	5.2	4.4	5.0	3.2	3.3
1.0	1.5	0.7	1.4	0.5	0.7	0.7
2.0	0.1	0	0	0	0	0
			Strain 44			
0	9.3	9.7	8.9	9.0	7.5	—
0.5	2.8	2.5	3.4	2.7	0.6	—
1.0	0.1	2.4	1.8	2.0	1.4	—
2.0	0.2	0.2	1.2	0.2	0.3	—

Neither taurine nor sodium γ-hydroxy buterate appeared to be inhibitors worth further study because of the large size of the effective concentrations, and because the relatively poor reversibility by pantothenate indicated considerable nonspecificity. Ivanovics (1942*a* and 1942*b*) has shown, for bacteria able to make their own pantothenic acid, that salicylate acts as a specific inhibitor and interferes with the synthesis of the pantoyl moiety of the molecule. Our data on *C. septicum* suggest that a similar relationship holds.

Strain 59 Li A, which is unable to make its own pantothenate, is inhibited by sodium salicylate but in a way that is not influenced by the pantothenate, pantoate, or β-alanine concentration of the medium (figure 4 and table 7). Investigation of the length of the lag period and the logarithmic rate of growth indicated that they did not behave differently in this respect than the final amount of growth. Preliminary studies of the inhibition by salicylate of the growth of strain 44 in a medium devoid of tryptose suggested that the inhibition could be reversed by pantoate or pantothenate. These studies were repeated in a medium containing 20 mg tryptose per ml and hence always a small amount (0.28 μg per ml) of pantothenate. The results are shown in table 7. There appears to be a slight decrease in the salicylate inhibition in the presence of high concentrations of pantoate or pantothenate, although the reversal is not very great. About 1,000 μg of calcium pantothenate per ml is the maximum concentration that can be used. After such a concentrated solution in our medium is autoclaved, a slight precipitate is formed whose turbidity is subtracted from that measured after the growth of the bacteria.

DISCUSSION

It is significant that of the possible pantothenic acid antagonists which we have used, three, sodium γ-hydroxy buterate, *dl*-pantoic amide, and sodium *dl*-pantoyltaurine, were able to substitute for pantothenic acid and supported the growth of pantothenate-requiring *C. septicum.* One of these compounds, sodium *dl*-pantoyltaurine, has been used as a chemotherapeutic agent (McIlwain and Hawking, 1943). It is very possible that our pantoyltaurine and pantoic amide were contaminated with some of the pantoyl lactone used in their preparation. As little as between 0.5 and 1.0 μg pantoyl lactone per mg of analogue is all that would be needed for the effects we observed. It would be necessary, however, to assume an unreasonable degree of contamination with pantoyl lactone to explain the results of Stansly and Alverson (1946). These authors report an antagonism of the salicylate inhibition of the growth of *Escherichia coli* by pantoyltaurine. It is more probable that some hydrolysis of pantoyltaurine occurs yielding *dl*-pantoate and taurine (cf. Sarett and Cheldelin, 1945*a*). Such a hydrolysis of about 2 per cent of the pantoyltaurine would not release sufficient taurine to inhibit significantly the growth of strain 59 Li and would be consistent with the data we have obtained.

On the other hand, the pantothenate activity of sodium γ-hydroxy buterate cannot be similarly explained, for this compound was not made from pantoyl lactone and cannot be directly transformed into it. Apparently γ-hydroxy buterate has about one hundred-thousandth of the catalytic activity of pantothenate.

There have been many attempts to inhibit specifically the utilization of pantothenic acid in bacteria by the use of chemical analogues of that growth factor (Nielson, Hartelius, and Johansen, 1944; Nielson and Roholt, 1945; Drell and Dunn, 1946; Mead *et al.*, 1946; cf. Roblin, 1946, for earlier references). The specific activities of these compounds not only depend upon their chemical constitution but are correlated with the pantothenic acid requirement of the organism being inhibited. With some exceptions pantothenate-independent organisms are resistant to inhibition by pantothenate analogues. A possible explanation of this relationship is that suggested by McIlwain (1942), which assumes that pantothenate produced within the cell is more effective in reversing inhibition than pantothenate supplied from the outside. He has been able to show (1945) that pantoyltaurine prevents the binding of pantothenate into functional form. On the other hand, Stansly and Alverson (1946) suggest that the pantoate formed from such compounds as pantoyltaurine (or the pantoyl lactone they contain) may be sufficient to enable an organism which can synthesize pantothenate from the pantoyl moiety to produce sufficient pantothenate to prevent inhibition. However, McIlwain's explanation is in better accord with the finding of Snell (1941*a* and 1941*b*) that pantoyltaurine inhibited the growth of a yeast stimulated with pantothenic acid, but it was ineffective when the yeast grew on β-alanine. Yet Sarett and Cheldelin (1945*b*) did not observe this relationship in a strain of yeast which was inhibited by pantoyltaurine when grown either on β-alanine or pantothenic acid. At the present time, in the absence of much fundamental information such as the permeability of vitamins and analogues, it is difficult to apply a general interpretation to the distribution of pantoyltaurine resistance among bacteria. For example, Woolley and Collyer (1945) have shown that phenyl pantothenone inhibited all of the organisms tested, although the inhibition was reversed by pantothenic acid only in those organisms which required pantothenic acid as a growth factor. Again, both pantothenate-independent and pantothenate-dependent strains of *C. septicum* are resistant to pantoyltaurine. Since interference with pantothenate metabolism is antagonized by amino acids (Ivanovics, 1942; Woolley, 1946*b*), it is possible that the resistance of the two strains of *C. septicum* was due to the high concentration of amino acids in our culture medium.

Investigations of the inhibition of growth of microorganisms by the use of chemical analogues of the pantothenate precursor, β-alanine, have also been numerous. Snell (1941*a* and 1941*b*), for example, failed to secure the inhibition by taurine of the growth of *Saccharomyces cerevisiae* stimulated with β-alanine, and Nielson (1943) and Nielson *et al.* (1944) confirmed this observation, although Sarett and Cheldelin (1945*b*) did find 1 of 17 strains of yeast requiring pantothenic acid or β-alanine for growth which was susceptible to taurine inhibition when grown on β-alanine. In the presence of β-alanine, but not of pantothenic acid, it was found that asparagine (cf. Weinstock *et al.*, 1939, and Atkin *et al.*, 1944) and many *l*-amino acids and natural products would inhibit yeast growth. These inhibitions were reversed by high concentrations of β-alanine. The same relations were found by Nielson and Johansen (1943) and Nielson *et al.* (1944) in the inhibition of yeast growth by many α-amino acids, isoserine, β-phenyl-β-alanine, and β-aminobutyric acid, and by Hartelius (1943) in the inhibition of yeast respiration by β-aminobutyric acid. In *Acetobacter suboxydans*, which requires pantothenic acid or the pantoyl moiety for growth, Sarett and Cheldelin (1945*a*) found that *dl*-alanine inhibited growth in the presence of pantothenic acid. Taurine inhibited the growth of this form more in the presence of the pantoyl moiety than in the presence of intact pantothenate, and the inhibition was completely overcome by excess β-alanine. These results suggest that the analogues inhibit by competing with β-alanine and hence interfering with the synthesis of pantothenic acid.

In *C. septicum* β-aminobutyric acid did not inhibit the growth of either the pantothenate-dependent or pantothenate-independent strains even when it was present in high concentrations. On the other hand, taurine did inhibit the growth of the pantothenate-dependent strain but only when present in very high concentrations. On this account and because pantothenate and β-alanine were so inefficient in their reversal of the inhibition, it is likely that most of the activity of taurine was nonspecific. Nevertheless, the failure of pantoate to antagonize taurine inhibition and the very slight antagonism shown by pantothenate and β-alanine suggest that part of the activity of taurine may be due to its interference in pantothenate synthesis through its competition with β-alanine. In the case of the inhibition of the growth of the pantothenate-independent strain of *C. septicum* by γ-hydroxy buterate, high concentrations were also necessary. Nevertheless, pantothenic acid was able to reverse this inhibition partially. If γ-hydroxy buterate interfered with the synthesis of pantothenate by competing with the pantoyl moiety, we would expect pantothenate to reverse the inhibition and β-alanine to be relatively ineffective. This was realized. We would also expect pantoate to reverse the inhibition. In the concentrations used such a reversal did not occur. Probably only the *d*-pantoate could be suspected of activity. Moreover, on a molar basis calcium *d*-pantothenate is about 20 times times as active as sodium *d*-pantoate in supporting the growth of *C. septicum*, and even more so when activity is calculated in terms of undissociated molecules (Ryan *et al.*, 1945). A further increase in the pantoate concentration may have resulted in a reversal. However, once again, inhibition occurred only in the presence of high concentrations of γ-hydroxy buterate and was not completely reversed by very large concentrations of pantothenate. Probably a large part of the inhibition is nonspecific in nature, though some of the inhibition is due to the specific interference with pantothenic synthesis.

Roblin (1946) has suggested that when an inhibitor affects the synthesis of a metabolite, the "minimum effective concentration of the metabolite which prevents the action of the antagonist is effective against any concentration of the latter." In the case of the inhibition of *C. septicum* by γ-hydroxy buterate the continued effect of increasing concentrations of this compound in the presence of high concentrations of pantothenate is probably due to the fact that its action is primarily nonspecific. If, on the other hand, as Nielson *et al.* (1944) propose, the action of β-alanine analogues is due to the fact that they become coupled with the pantoyl moiety to form inactive derivatives of pantothenic acid, then the suggestion of Roblin would not apply.

Our results on the inhibition of the growth of *C. septicum* by salicylate are in general agreement with the conclusions reached by Ivanovics (1942*a* and 1942*b*) and Stansly and Schlosser (1945). The pantothenate-independent strain was inhibited by salicylate, and this inhibition was partially prevented by pantoate and pantothenate but not by β-alanine. Such a relationship would be expected if salicylate were interfering with pantothenate synthesis by competition with the pantoyl moiety. The pantothenate-dependent strain should then be inhibited by salicylate only in high concentrations and then in a way that is not reversed by either pantothenate or its split products. The salicylate inhibition of the pantothenate-dependent strain of *C. septicum* was not reversed by these substances. However, the concentration of salicylate required for the complete inhibition of the pantothenate-independent strain is only about one-half of that required for the complete inhibition of the pantothenate-dependent strain (about 0.01 M). Ivanovics was able to show that amino acids could eliminate the specific antivitamin action of salicylate. Perhaps in the case of the pantothenate-independent strain of *C. septicum* the presence of casamino acids (and of tryptose) in the medium antagonized much of the specific inhibitory action of salicylate. The inhibition observed may be largely nonspecific and hence resemble the salicylate inhibition of the pantothenate-dependent strain. These data then do not disagree with the hypothesis that salicylate can interfere with pantothenate synthesis by competing with the pantoyl moiety.

None of the inhibitors described in this paper were successful as chemotherapeutic agents against experimental gas gangrene in mice (Ryan *et al.*, 1946).

SUMMARY

Two strains of *Clostridium septicum* were used in this study. One is unable to synthesize the pantoyl moiety of the pantothenate molecule and hence, for growth, requires either of these substances in the medium. The other strain can make, and is independent of an external supply of, these substances.

An improvement of Bernheimer's chemically defined medium is described. An examination is made of the role of several factors, such as sterile filtration and natural products like tryptose, in increasing growth.

Several chemical analogues of β-alanine, of the pantoyl moiety, and of pantothenate were chosen as possible antivitamins. Of these only two were active, sodium γ-hydroxy buterate inhibiting the pantothenate-independent strain and taurine inhibiting the pantothenate-dependent strain. In addition, sodium salicylate inhibits growth in a way that suggests that it interferes with the synthesis of the pantoyl moiety of the pantothenate molecule.

Three of the analogues, sodium *dl*-pantoyltaurine, sodium γ-hydroxy pantoate, and pantoic amide, have pantothenate activity for the pantothenate-dependent strain.

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THE NUTRITION OF PROTOZOA

I. A SIMPLIFIED MEDIUM FOR THE INVESTIGATION OF UNKNOWN FACTORS IN BLOOD SERUM ESSENTIAL FOR THE SUSTAINED GROWTH OF TRICHOMONAS VAGINALIS[1]

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Media devised for the cultivation of parasitic protozoa almost invariably require blood serum at the very least (Greene, 1945; Senekjie and Lewis, 1945; Ball *et al.*, 1945), if not also other blood constituents, as was supposed by earlier workers. Very little is known at present about the factors in serum necessary for the growth and maintenance of these organisms.

It has recently been demonstrated by Johnson and R. E. Trussell (1943) that the protozoan *Trichomonas vaginalis* can be grown and maintained in pure culture in a basal medium composed chiefly of Difco peptone, liver infusion, maltose, and cysteine, to which unheated blood serum must be added. Moreover, Johnson and M. H. Trussell (1945) have shown that liver infusion may be completely replaced by ascorbic acid, glutamic acid, choline chloride, folic acid, and xanthopterin. The luxuriant growth obtained with this medium in 36 to 48 hours, the low-grade pathogenicity of the organism, and the ease with which it can be handled *in vitro* are all factors which combine to render *Trichomonas vaginalis* admirably suitable as an assay organism for determining the chemical components of blood serum necessary for the growth and maintenance of a parasitic flagellate.

Before serum fractions could be tested with any degree of rapidity and assurance, however, it was necessary to devise a basal medium for *Trichomonas vaginalis* which could be made up in a minimum of time, which could be rendered complete with respect to serum or fractions prepared therefrom with a maximum of convenience, and whose composition was reasonably well defined from a chemical standpoint.

The present paper presents in detail the preparation of stock solutions and the compounding of a final medium from these stock solutions in order to obtain a culture fluid which will meet the requirements mentioned above. This medium has been used as a starting point in an attempt to devise a chemically defined medium for the sustained growth of *Trichomonas vaginalis*. Experiments dealing with fractionation of blood serum will be described in subsequent papers.

EXPERIMENTAL

Organism used. The organism used was a bacteria-free culture of *Trichomonas vaginalis* strain no. 2 which was isolated in March, 1945, from a patient with a

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severe vaginitis (Johnson, Trussell, and Jahn, 1945). Batteries of stock cultures were carried at 37 C in the revised medium described below and transferred every 48 to 72 hours. The inoculum used to assay serum fractions was prepared from a 48- to 72-hour culture which was centrifuged aseptically, washed twice, and resuspended in sterile Ringer's solution in a volume approximating three-fourths that of the original culture fluid. Then 0.03 to 0.05 ml (approximately 80,000 organisms) were introduced by pipette at the midlevel of the assay tube. Serial transfers of 0.03 to 0.05 ml of inoculum were made every 48 to 72 hours. Growth was measured by hemocytometer cell counts at the end of a 48- to 72-hour period.

Basal medium. The serum-free basal medium used was a modification of the original CPLM culture fluid devised by Johnson and Trussell (1943). In the revised medium described below, the Difco peptone was replaced by "trypticase" (Baltimore Biological Laboratories) and the liver infusion by a mixture of B vitamins, purines, and pyrimidines. In addition, acetate, asparagine, ascorbic acid, bicarbonate, and ribose were added.

Attempts to replace the Difco peptone by vitamin-free casein hydrolyzate (General Biochemicals, Inc.) fortified with cystine, tryptophane, and glycine were unsuccessful. Difco peptone, however, could be replaced by trypticase. The use of trypticase has the advantage of defining more clearly than Difco peptone the nature of the nitrogenous requirements of the organism, since trypticase is known to be a pancreatin digest of casein and contains no detectable carbohydrate.

No attempt has been made to limit the B vitamins, purines, pyrimidines, or the other pure chemical compounds added to a basis of essentiality. Rather, it was felt, the medium should be made as complete as possible with respect to *known* growth factors so as to rule these out in assays of serum fractions for the unknown substances necessary for the growth of *Trichomonas vaginalis.*

The constituents of the medium were prepared as follows:

(1) *Stock B vitamin solution.* The following B vitamins were suspended in 100 ml of 20 per cent alcohol: 20 mg of thiamine hydrochloride, 80 mg of pyridoxine hydrochloride, 20 mg of pyridoxamine hydrochloride, 20 mg of pyridoxal hydrochloride, 40 mg of calcium pantothenate, 100 mg of riboflavin, 40 mg of nicotinic acid, and 20 mg of *p*-aminobenzoic acid. The mixture was stored in the cold and shaken well before using.

(2) *TV stock solution I.* The following ingredients were dissolved in 1,000 ml of the modified Ringer's solution used in the CPLM medium (NaCl, 0.6 per cent; $NaHCO_3$, $CaCl_2$, KCl, 0.01 per cent) to give a double-strength stock solution: 2.5 g of maltose, 12.0 g of sodium acetate, 0.5 g of asparagine, 20 mg of choline chloride, 20 mg of inositol, 10 mg of ribose, 2 ml of *stock B vitamin solution,* 200 μg of biotin, and 200 μg of folic acid. This stock solution was kept in the cold under toluene.

(3) *TV stock solution II.* The following purines and pyrimidines were dissolved in 700 ml modified Ringer's solution: 50 mg of adenine SO_4, 50 mg of guanine HCl, 50 mg of xanthine, and 50 mg of uracil. One ml of 5 N NaOH was added, the suspension was shaken well, and 0.1 N NaOH was added drop by drop

until a clear solution resulted. The final volume was adjusted with Ringer's solution to 1,000 ml. This solution was likewise kept in the cold under toluene.

(4) *Sterile ascorbic acid sodium bicarbonate stock solution.* Two and five-tenths g of sodium bicarbonate were suspended in 40 ml of H_2O; 0.5 g of ascorbic acid dissolved in 10 ml of H_2O were added slowly with gentle stirring. This gave 50 ml of a 1 per cent ascorbic acid and 5 per cent sodium bicarbonate solution, which was then sterilized by Seitz filtration. This solution was prepared and added immediately before assay to prevent excessive exposure of ascorbic acid to an alkaline pH.

(5) *Methylene blue stock solution.* A stock solution of 0.5 per cent methylene blue in distilled water was used as described below to indicate the degree of anaerobiosis in the final medium. Its addition may be omitted.

TABLE 1

*Composition of complete trypticase nutrient medium for Trichomonas vaginalis**

(The amounts listed are the calculated values per 10 ml of final medium)

Trypticase (BBL)	200 mg	Riboflavin	8.0 μg
Sodium acetate $3H_2O$	48 mg	Thiamine HCl	1.6 μg
Cysteine HCl	15 mg	Pyridoxine HCl	6.4 μg
Maltose	10 mg	Pyridoxamine HCl	1.6 μg
Difco agar	10 mg	Pyridoxal HCl	1.6 μg
$NaHCO_3$†	5 mg	Ca pantothenate	3.2 μg
Asparagine	2 mg	Nicotinic acid	3.2 μg
Ascorbic acid†	1,000 μg	*p*-Aminobenzoic acid	1.6 μg
Choline chloride	80 μg	Biotin	0.8 μg
Inositol	80 μg	Folic acid	0.8 μg
Ribose	40 μg	Methylene blue (optional)	24.0 μg
Adenine SO_4	40 μg	Adjusted to pH 6.0, and Ringer's solution added to make:	8.0 ml
Guanine HCl	40 μg	Human blood serum diluted with equal volume of Ringer's solution†	2.0 ml
Xanthine	40 μg	Final volume	10.0 ml
Uracil	40 μg		

* To render the medium basal, serum is omitted.

† Added as sterile solutions after the medium was autoclaved.

To prepare 500 ml of the trypticase basal medium (so called in contrast to the CPLM medium), the following mixture was made: 250 ml of TV stock solution I, 50 ml of TV stock solution II, and 625 mg of Difco agar were brought to a boil in a 400-ml beaker heated over a steam bath. Heating was continued until the agar was completely dissolved. The solution was filtered while hot through porous Reeve-Angel filter paper. To the warm filtered mixture there were now added 0.3 ml of 0.5 per cent methylene blue, 12.5 g of trypticase (BBL), and 935 mg of cysteine hydrochloride. The mixture was stirred well, and mild heat was applied until a perfectly clear solution resulted. After being cooled to room temperature the mixture was adjusted to pH 6.0 with 1 N HCl or 1 N NaOH and checked by the glass electrode. Finally the solution was diluted to 500 ml with the modified Ringer's fluid, tubed in 8-ml lots, autoclaved, and allowed to cool.

Then 0.1 ml of the sterile ascorbic acid sodium bicarbonate solution was added aseptically to each tube. Tubes thus prepared may be stored at room temperature for a period of 2 weeks without any deterioration of the medium.

To render the medium complete, blood serum was diluted with an equal volume of Ringer's solution and passed through a sterile filter (Berkefeld or Seitz). Two ml of this sterile diluted serum were then added aseptically to each tube containing 8 ml of the basal Trypticase medium, giving a final volume of 10 ml.

The composition of the final complete trypticase nutrient medium for the cultivation of *Trichomonas vaginalis* is given in table 1.

Assay of serum fractions may now be accomplished by adding in place of the intact serum any one given fraction or combination of fractions to a tube containing 8 ml of the trypticase basal medium. As much as 2 ml of the material to be tested may be used. Whenever volumes less than 2 ml are employed, Ringer's solution must be added to compensate for the difference. The final volume of each tube should always be 10 ml to permit a correct cell count comparison with intact serum controls.

TABLE 2

Growth of Trichomonas vaginalis in trypticase medium compared with that in CPLM medium

NUMBER OF SERIAL TRANSFER	GROWTH OF TRICHOMONAS VAGINALIS EXPRESSED IN NUMBER OF CELLS PER MM^3 AFTER 48 HOURS IN EACH SERIAL TRANSFER*	
	8 ml of trypticase basal + 2 ml of diluted serum†	8 ml of CPLM basal + 2 ml of diluted serum†
First culture	1,180	2,275
Serial transfer no. 1	1,190	2,500
2	1,550	2,185
3	1,170	1,055
4	915	1,170
5	1,565	2,450
6	1,040	2,200
7	1,135	2,500
8	1,565	3,360
9	1,655	2,275
10	1,580	1,450
11	1,585	1,140

* Each value is the average of duplicate determinations.

† The serum was diluted with an equal volume of Ringer's solution before assay.

RESULTS AND COMMENTS

The ability of this new trypticase medium to maintain sustained growth of *Trichomonas vaginalis* as compared with the original CPLM culture fluid is evident from table 2.

Growth through 11 successive subcultures is presented in table 2. To date, over 60 serial transfers have been made, and sustained growth has been well maintained. Although the number of cells present after 48 hours in the trypticase medium was appreciably less than in the CPLM culture fluid, the organisms were more uniform in shape and larger in size, and possessed a greater degree of motility. The higher cell count obtained with the CPLM medium was due to the presence of stimulatory factors in the liver infusion used in that preparation. The same effect could be obtained with the trypticase medium if the B vitamins, purines, and pyrimidines were replaced by 0.15 per cent of Wilson's liver fraction L. In one experiment in which this was done, an average count of duplicate determinations came to 2,885 cells per mm^3 on the seventh serial transfer.

Attempts to omit agar from the basal medium have resulted in irregular growth. Whether the stimulatory effects of agar are mechanical or chemical is still being investigated.

The cell count method of measuring growth is time-consuming and tedious. Some attempts have been made to measure growth by a turbidimetric procedure involving the use of the Evelyn photoelectric colorimeter. It is possible that this technique may become practicable in future investigations.

SUMMARY

The original basal CPLM medium of Johnson and Trussell has been modified to make it suitable for the assay of unknown growth factors in blood serum essential for the sustained growth of *Trichomonas vaginalis* in pure culture. The Difco peptone was replaced by trypticase (BBL) and the liver infusion by a mixture of B vitamins, purines, and pyrimidines. In addition, acetate, asparagine, ascorbic acid, bicarbonate, and ribose were added. This medium has the advantage of being better defined chemically than the CPLM medium, and has been devised for convenient preparation from stock solutions in a minimum of time.

Although growth, measured by the number of cells per cubic millimeter, was appreciably less in 48 hours in the trypticase culture fluid than in the CPLM medium, nevertheless sustained growth in the trypticase medium has been maintained through 60 serial transfers. Furthermore, the organisms in the trypticase nutrient were more uniform in shape and larger in size, and possessed a greater degree of motility.

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THE NUTRITION OF PROTOZOA

II. THE SEPARATION OF HUMAN BLOOD SERUM INTO TWO FRACTIONS, BOTH ESSENTIAL FOR THE SUSTAINED GROWTH OF TRICHOMONAS VAGINALIS[1]

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In the preceding paper (Sprince and Kupferberg, 1946) it was pointed out that very little is known about the factors in blood serum necessary for the sustained growth of parasitic flagellates. The advantages of using *Trichomonas vaginalis* as an assay organism in investigating such factors were discussed. A detailed description of a basal medium, which was well defined chemically and was devised primarily for rapid and convenient assay of such factors in serum, was presented.

It is the purpose of this paper to report that human blood serum can be separated into two fractions— (1) an ether-soluble fraction, and (2) the remaining ether-insoluble residue—both of which are necessary for the sustained growth of *Trichomonas vaginalis.*

EXPERIMENTAL

Organism used. The organism used was a pure culture of *Trichomonas vaginalis* strain no. 2. The history of this organism, the manner in which it has been carried, and the preparation of the inoculum prior to assay have been described in the first paper of this series.

Basal medium. The trypticase basal medium used below and the assay procedure followed have been described in detail in the same publication.

Fractionation of serum. Serum fractions were prepared as follows: 100 ml of human blood serum[2] (pooled from negative Wassermann tests) were adjusted to pH 7.1 to 7.3 with several drops of either N HCl or N NaOH and checked with the glass electrode. The serum was then extracted with 200 ml of ethyl ether in a separatory funnel at room temperature. Eight to twelve extractions were made in this manner, the number being determined by lack of further color extractable by the ether. The pH was readjusted after each extraction to 7.1 to 7.3, and the volume of the aqueous phase was kept at approximately 100 ml to 125 ml.

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[1] We wish to thank Dr. Jordi Folch-Pi of the Rockefeller Institute Hospital, New York City, for a sample of his plasma lipid fraction. For small quantities of human serum albumin, we are indebted to Dr. John Edsall of the Department of Physical Chemistry, Harvard Medical School. Human blood serum from negative Wassermann tests was made available to us through the kindness of Mr. J. H. Spooner of the State Department of Health, Trenton, New Jersey.

We are grateful to Dr. Garth Johnson for his generous counsel and encouragement in this investigation.

[2] The serum was heated to 55 C for 15 minutes, as is usually done in routine Wassermann tests to destroy the natural complement.

The ether extracts were pooled, and one-fourth of the total volume (∞ 25 ml intact serum) was evaporated to dryness *in vacuo*. Five ml of distilled water were added and evaporated to dryness twice. Finally 22 ml of distilled water were added. The mixture was now adjusted to pH 7.1 to 7.3 and shaken vigorously for several hours. The emulsion formed was readjusted to pH 7.1 to 7.3, brought to 25-ml volume with distilled water (thereby equating it to serum in concentration), and sterilized by being filtered through a sterile Seitz or fritted glass filter (Corning GYVKU). Use of the Corning glass filter necessitated constant stirring with a rubber policeman. The remaining ether extract was kept in the cold room for future use.

The ether-insoluble phase was reduced by evaporation *in vacuo* at a temperature under 45 C to a volume at least 10 ml beyond the point of detectable ether odor. Distilled water was then added to give a volume of 100 ml (to bring to serum concentration), and the solution was sterilized by filtration as described above.

Several points in this separation procedure should be noted. Prolonged, vigorous shaking of the separatory funnel should be avoided, since a third layer may form between the ether and aqueous phases that consists of a viscous mixture of ether, water, and serum material. This often persisted even after standing overnight. In every case in which such a layer formed, it was regarded as belonging to the ether-insoluble phase and separations were made accordingly. Obviously, the addition of a sizable portion of third layer during the separation procedure to the ether-insoluble phase may introduce enough of the ether-soluble phase to render the former phase active per se when it is subjected to assay. Great care should be taken to avoid this.

At best, the sterile filtrations were long and tedious. Occasionally, a complete block of the filter would be encountered. In such cases, filtrations were effected by making up the fraction in question as a 5 per cent alcohol solution. For assay purposes, all serum fractions must be homogeneous, and must remain so when mixed with the basal medium.

Ether fractionation of serum below freezing temperatures has been reported to result in excellent extraction of lipoidal material (McFarlane, 1942). For our purposes, however, such attempts proved disappointing. Fractions were obtained which did not emulsify satisfactorily with water, and consequently did not lend themselves to subsequent sterile filtration. In this connection, see Cohn *et al.* (1946, footnotes 25 and 72). It is possible, however, that by the use of proper protective agents, stable emulsions may be obtained.

The preparation of serum fractions for assay. It will be noted that the sterile serum fractions were made up in volumes equivalent to their original concentration in serum. As indicated in the preceding paper (Sprince and Kupferberg, 1946), serum controls were always diluted with an equal volume of Ringer's solution and sterilized by filtration prior to assay. Consequently, all serum fractions equal to serum in concentration were likewise diluted with an equal volume of Ringer's solution and filtered sterilely before assay. This was done to permit the comparison of the potency of a given fraction in replacing serum.

Data for a typical experiment in which serum has been replaced by fractions prepared therefrom are given in table 1.

From table 1 it is evident that neither the aqueous emulsion of the ether extract nor the ether-insoluble phase *alone* supported growth. Even doubling the volume of the separate fractions added to the basal medium gave no growth response. Addition of the two components together resulted in sustained growth through 5 serial transfers in numbers comparable to 5 serial transfers with intact serum. The results presented above have been repeated with 5 different lots of pooled serum.

The ether-soluble phase. Attempts have been made to determine the nature of the active material in the ether-soluble phase.

TABLE 1

In vitro cultivation of Trichomonas vaginalis in fractions prepared from human blood serum

ML OF DILUTED SERUM CONTROL* OR DILUTED SERUM FRACTION* ADDED TO 8 ML OF TRYPTICASE BASAL MEDIUM TO GIVE A TOTAL VOLUME OF 10 ML PER TUBE	GROWTH OF TRICHOMONAS VAGINALIS EXPRESSED IN NUMBER OF CELLS PER MM^3 AFTER 48 HOURS IN EACH SERIAL TRANSFER†					
	First culture	Serial transfer 1	Serial transfer 2	Serial transfer 3	Serial transfer 4	Serial transfer 5
Control (2 ml of diluted intact serum)	1,550	1,040	740	1,895	1,305	1,510
1.0 ml diluted aqueous emulsion of ether extract + 1.0 ml sterile Ringer's	0					
1.0 ml diluted ether-insoluble phase + 1.0 ml sterile Ringer's	990	540	0			
1.0 ml diluted aqueous emulsion of ether extract + 1.0 ml diluted ether-insoluble phase	1,080	685	1,205	2,335	1,755	1,145

* All samples of intact serum or serum fractions were diluted with an equal volume of Ringer's solution, as indicated in a preceding paragraph.

† Each value is the average of duplicate determinations.

(1) *The effect of heat.* One ml of diluted aqueous emulsion of ether extract (≎ 0.5 ml serum) was added to each of duplicate tubes containing trypticase basal medium before autoclaving. The tubes were then autoclaved and cooled, and 1.0 ml of the sterile diluted ether-insoluble phase was introduced. Sterile ascorbic acid sodium bicarbonate solution was now added as usual, and the tubes were assayed.

(2) *Dialysis of aqueous emulsion of ether extract.* Ten ml of undiluted aqueous emulsion of ether extract (≎ serum concentration, and pH adjusted to 5.8) was dialyzed against six 100-ml portions of distilled water over a period of 1 week at 4 C (to prevent microbial contamination). The dialyzate was then evaporated to dryness *in vacuo,* and the residue was made up to the original volume (10 ml) with distilled water and adjusted to pH 6.8. The homogeneous solution was

then diluted with 10 ml of Ringer's solution and sterilized by filtration. One-ml samples of this material were now introduced into duplicate tubes containing 8.0 ml sterile trypticase basal medium to which there had already been added 1.0 ml of sterile diluted ether-insoluble phase plus sterile ascorbic acid bicarbonate solution. Assays proceeded in the usual manner.

(3) *Replacement experiments.* The following amounts of representative fat-soluble compounds of physiological significance were dissolved in 50 to 100 ml of ether: 60 mg of lecithin, 30 mg of cholesterol, 30 mg of oleic acid, 30 mg of linoleic acid, 30 mg of ergosterol, 3 mg of α-estradiol, 300 μg of α-tocopherol, 60 μg of β-carotene, and 60 μg of vitamin A. The ether solution was evaporated to dryness, and the residue was made up in distilled water, adjusted to pH 7.2, and diluted to 30.0 ml. After dilution with Ringer's solution to 60 ml and sterile filtration, assays with 1.0-ml samples were undertaken as described previously.

TABLE 2

The nature of the material in the ether-soluble phase essential for the sustained growth of Trichomonas vaginalis

FRACTION REPRESENTING ETHER-SOLUBLE PHASE ADDED*	GROWTH OF TRICHOMONAS VAGINALIS EXPRESSED IN NUMBER OF CELLS PER MM³ AFTER 48 HOURS IN EACH SERIAL TRANSFER†					
	First culture	Serial transfer 1	Serial transfer 2	Serial transfer 3	Serial transfer 4	Serial transfer 5
None (intact serum, control)	1,040	1,140	1,565	1,655	1,580	1,585
None (aqueous ether-insoluble phase, control)	990	0				
Autoclaved aqueous emulsion of ether extract	1,660	1,555	1,985	1,110	665	1,005
Dialyzate of aqueous emulsion of ether extract	2,075	1,340	1,895	1,695	1,600	1,640
Replacement mixture of pure compounds (described above)	710	1,135	450	950	1,560	1,120

* All fractions equivalent to serum in concentration were diluted with an equal volume of Ringer's solution. One ml of the diluted material was then assayed in test tubes containing 8.0 ml of trypticase basal medium plus 1.0 ml of diluted ether-insoluble phase.

† Each value is the average of duplicate determinations.

The results obtained in these studies of the nature of the active material in the ether extract are summarized in table 2.

It is readily apparent that the active material in the ether-soluble phase is heat-stable and dialyzable, and can be replaced rather effectively by a homogenized aqueous emulsion of the mixture of compounds described above. A plasma lipid fraction prepared by Doctor Jordi Folch-Pi of the Rockefeller Institute Hospital, New York City, also proved to be quite active.

Preliminary experiments have indicated that the active constituent in the mixture is linoleic acid. In one instance in which linoleic acid (Eimer and Amend) was used in place of the ether-soluble phase, an average count of duplicate determinations on the fifth serial transfer came to 955 cells per mm³ as compared with 1,420 cells per mm³ with intact serum controls. As will be seen below, present indications point to the passage of other stimulatory factors, in addition to linoleic acid or the mixture described above, into the ether extract during the separation procedure.

The ether-insoluble phase. Experiments were performed in which the ether-insoluble phase was replaced by a solution of human serum albumin.

A 5 per cent solution of human serum albumin (approximate concentration in human serum) was made up in distilled water and diluted with an equal volume of Ringer's solution for assay purposes. In each experiment described below, 1.0-ml samples of this preparation were assayed in tubes containing 8 ml of the usual trypticase medium to which 1 ml of a properly diluted fraction representing the ether-soluble phase was added.

TABLE 3

Replacement of ether-insoluble phase by human serum albumin

FRACTION REPRESENTING ETHER-SOLUBLE PHASE ADDED*	GROWTH OF TRICHOMONAS VAGINALIS EXPRESSED IN NUMBER OF CELLS PER MM³ AFTER 48 HOURS IN EACH SERIAL TRANSFER†					
	First culture	Serial transfer 1	Serial transfer 2	Serial transfer 3	Serial transfer 4	Serial transfer 5
None (intact serum, control)	1,180	1,170	1.170	915	1,565	1,655
None (human serum albumin, control)	900	0				
Aqueous emulsion of ether extract	355	460	615	450	395	335
Replacement mixture of pure compounds (described above)	570	0				

* All fractions equivalent to serum in concentration were diluted with an equal volume of Ringer's solution. One ml of the diluted material was then assayed in test tubes containing 8.0 ml of trypticase basal medium plus 1.0 ml of diluted human serum albumin.

† Each value is the average of duplicate determinations.

Table 3 presents data to demonstrate that the ether-insoluble phase can be replaced to some extent by human serum albumin if the ether extract component of serum is present in the medium. Sustained growth could be maintained under these conditions, but the resulting cell count was considerably less. Increasing the concentration of the ether extract and of the human serum albumin twofold did not effect an increased cell count. Evidently stimulatory factors normally present in the ether-insoluble fraction were lacking under these circumstances.

Attempts to replace the ether extract of serum with the mixture of pure compounds (lecithin, cholesterol, oleic acid, linoleic acid, ergosterol, α-estradiol, α-tocopherol, β-carotene, and vitamin A) in a medium containing human serum albumin in lieu of the aqueous ether-insoluble phase were unsuccessful. This would indicate that substances other than these compounds (including linoleic acid) were present in the ether extract of serum which contributed to its activity.

DISCUSSION

The separation of blood serum into two components, (1) ether-soluble and (2) ether-insoluble, both of which are necessary for the growth of a parasitic flagellate, is an observation which is of interest in light of previous investigations. In contrast to the findings of Cailleau for *Trichomonas columbae* (1936), cholesterol was not the active factor in the ether-soluble extract. Moreover, in confirmation of Kupferberg and Johnson (1941), estradiol was also found to be inactive. On the other hand, it should be noted that a number of fatty acids have been reported as growth accelerants for flagellates (Lwoff and Provasoli, 1937; Provasoli, 1937). In view of these findings and our own observation with linoleic acid, a reinvestigation of both saturated and unsaturated fatty acids as possible growth factors for parasitic flagellates might be in order.

The recent findings of Senekjie and Lewis (1945) are of particular interest. These investigators were able to demonstrate the existence of a factor in blood serum essential for the cultivation of leishmanias and trypanosomes. This active principle was dialyzable but not directly associated with albumin, euglobulin, pseudoglobulin, or any combination of these fractions. It is interesting to note that our factor in the ether extract of serum active for *Trichomonas vaginalis* was also dialyzable. It differed from the Senekjie-Lewis factor in being stable to autoclaving, whereas the latter factor survived heating at 70 C for 30 minutes only partially and was completely destroyed at 100 C. Nevertheless, the possibility that the active factor (or factors) present in the ether-soluble extract of our experiments is identical with that of Senekjie and Lewis should be considered.

SUMMARY

Human blood serum has been separated by ether extraction into two fractions, both of which are necessary for the sustained growth of *Trichomonas vaginalis* in trypticase basal medium. The fractions are (1) an ether-soluble fraction and (2) the aqueous ether-insoluble residue remaining after the extraction. Neither fraction alone supports growth, but addition of both to the basal culture fluid provides a complete nutrient medium. Preliminary experiments indicate that one of the active components of the ether-soluble fraction is linoleic acid (Eimer and Amend, pure). Serum albumin is one of the active components of the aqueous ether-insoluble phase. Factors necessary to effect optimal growth of *Trichomonas vaginalis* exist in both fractions, but the nature of these is, at present, unknown.

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STUDIES ON THE MORPHOLOGY OF THE ELEMENTARY BODIES OF FOWL POX

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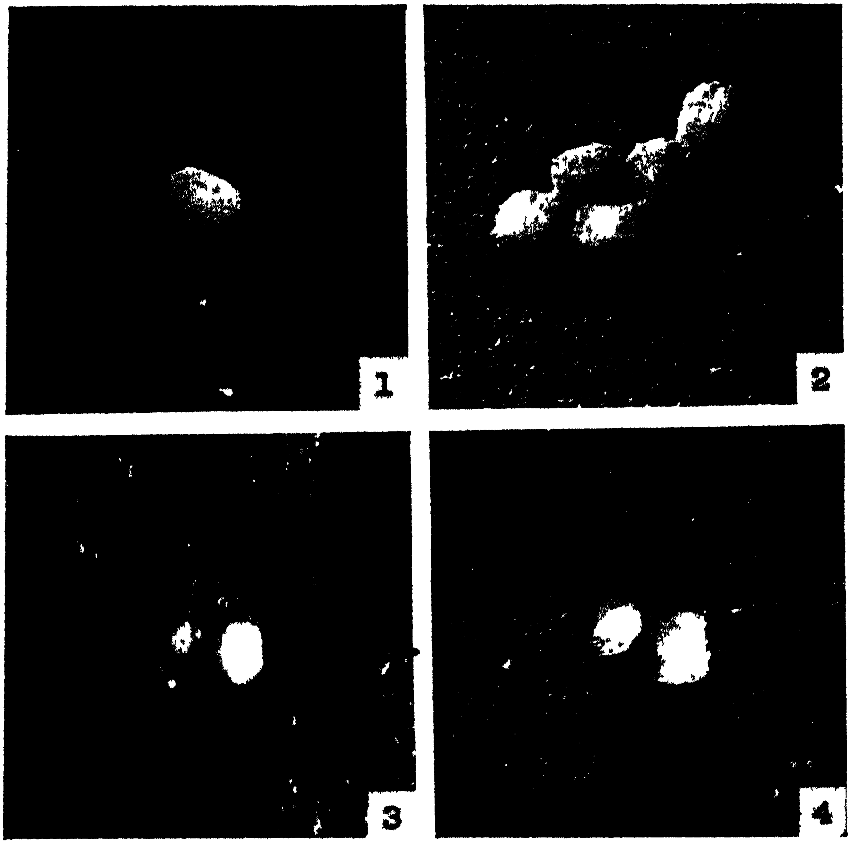
The morphological structure of the elementary bodies of vaccinia as revealed by the electron microscope was first studied by Green, Anderson, and Smadel (1942). Pictorial data presented by these workers showed that the elementary bodies are roughly rectangular in shape, possess a limiting membrane, and exhibit rounded areas of density greater than that of the remainder of the particle, suggestive of internal differentiation. These findings were confirmed by Sharp, Taylor, Hook, and Beard (1946), who, in addition, utilized the shadow-casting technique of Williams and Wyckoff (1945) and presented evidence that the elementary bodies are essentially short cylinders apparently coated with a sticky substance. Recently Groupé, Oskay, and Rake (1946) have shown that the elementary bodies of fowl pox closely resemble those of canary pox and have many morphological characteristics in common with the elementary bodies of vaccinia. In addition, it was observed that the particles of these avian poxes are most frequently attached to one another at their corners, and that the characteristically flattened corner observed on many particles probably results from the separation of particles thus joined. In the studies presented below, the shadow-casting technique of Williams and Wyckoff (1945) has been utilized to study further the morphological characteristics of the elementary bodies of fowl pox with particular reference to their internal structure and the curious budlike structures previously described (Groupé, Oskay, and Rake, 1946).

MATERIALS AND METHODS

An egg-adapted strain of fowl pox was obtained through the courtesy of Dr. F. R. Beaudette of the New Jersey Experiment Station and was maintained by serial egg passage using Burnet's technique (Burnet, 1936). Heavily infected chorioallantoic membranes were ground with sand and 10 per cent suspensions in saline prepared. Partial purification of the elementary bodies was obtained by two cycles of differential centrifugation at speeds of 1,000 rpm and 15,000 rpm for 15 minutes and 45 minutes respectively. The pellet obtained from the final high-speed sedimentation of virus was resuspended in distilled water, and a small drop of the resulting virus suspension was immediately placed on the collodion film support. After drying in air the specimens were ready for shadow casting. We are greatly indebted to Dr. H. Sidney Newcomer, who designed and built the shadow-casting device used in these studies. This device has given excellent results in the application of the technique introduced by Williams and Wyckoff (1945). Satisfactory shadowing of the elementary bodies was obtained when about 20 mg of gold were vaporized, approximately 8 to 10 cm from the specimen, at an angle ranging from 8 to 15 degrees, under a vacuum of the order of 0.00003 mm Hg. An RCA electron microscope (type EMU) was used throughout these studies

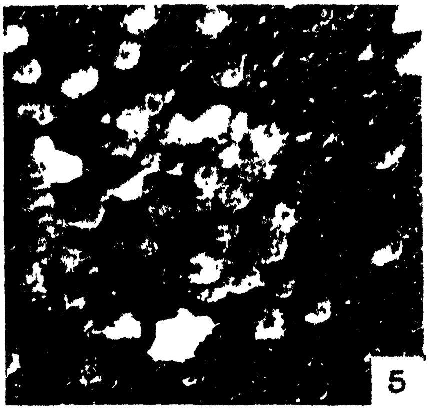


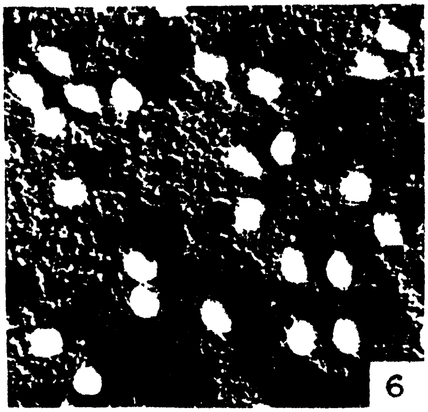
Figs 1–4 Fowl Pox Virus Shadowed with Gold

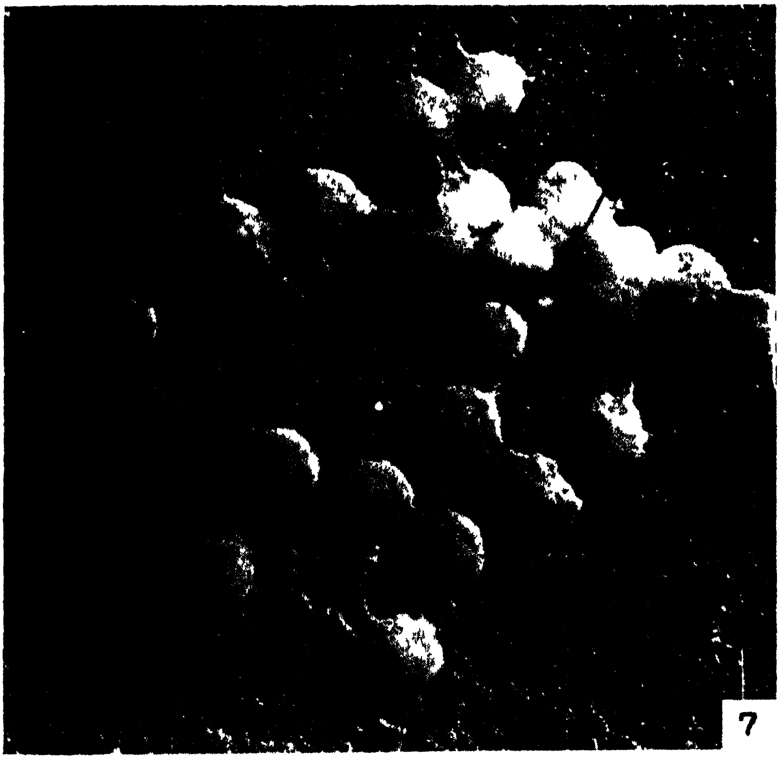
1 Shadowed with 20 mg of gold at the angle tangent 1 3/9 0   2 4 × 12,400×
2 Shadowed with 19 mg of gold at the angle tangent 2/10   2 4 × 12,400×
3 Shadowed with 20 mg of gold at the angle tangent 1 3/9 0   2 4 × 8,620×
4 Shadowed with 19 mg of gold at the angle tangent 2/10   2 3 × 12,400×

EXPERIMENTAL

It will be seen (figures 1 and 2) that the elementary bodies of fowl pox appear to be flattened in varying degree and are usually rectangular in shape, although rounded forms are not uncommonly observed (figures 5, 6, and 7). It would seem that the particles settle on the collodion film with a certain amount of collapse similar to the behavior of the elementary bodies of vaccinia (Green, Anderson, and Smadel, 1942, Sharp, Taylor, Hook, and Beard, 1946) and feline pneumonitis (Rake, Rake, Hamre, and Groupé, 1946). Assuming the elementary bodies of fowl pox to be short cylinders like those of vaccinia (Sharp, Taylor,







FIGS. 5–7. FOWL POX VIRUS SHADOWED WITH GOLD

5. Shadowed with 20 mg of gold at the angle tangent 1.3/9.0. 2.4 × 5,900×.
6. Shadowed with 20 mg of gold at the angle tangent 1.3/9.0. 2.4 × 5,900×.
7. Shadowed with 19 mg of gold at the angle tangent 2/10. 2.3×12,400×.

Hook, and Beard, 1946), the presence of rounded forms could be explained by the manner in which the particles settle on the collodion film.

It has been noted by Groupé, Oskay, and Rake (1946) that the particles of fowl pox and canary pox are usually attached to one another at their corners and that the characteristically flattened corner frequently observed on many particles (figure 1) probably results from the separation of two such particles. A typical group of elementary bodies characteristically joined at their corners by an interparticular bridge is presented in figure 2. These characteristics are also evident in the micrographs of vaccinia virus of other investigators (Green, Anderson, and Smadel, 1942; Sharp, Taylor, Hook, and Beard, 1946). The curious budlike structures previously described, which usually also appear at the corners of the particles, were not infrequently encountered in shadowed preparations. Two typical examples are shown in figures 3 and 4 It will be seen that such structures vary considerably in size and are definitely connected with their respective elementary bodies. It would seem reasonable to suppose that these structures result from unequal division of the elementary bodies. Similar unequal division has been observed with the bacteria (Mudd and Anderson, 1944).

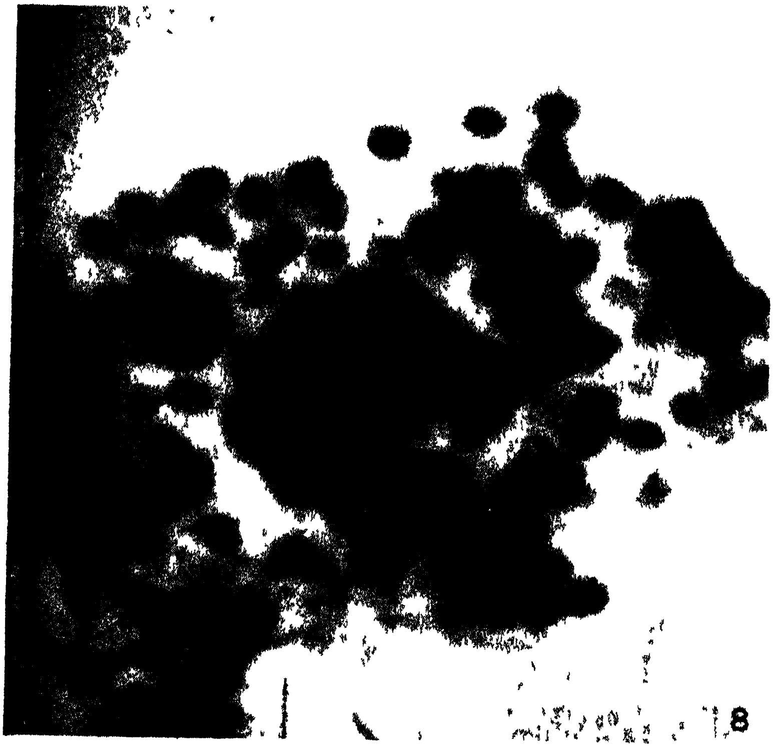


Fig 8 Fowl Pox Virus 2 4 × 5,900×

Inasmuch as the large central area of condensation in the particles of vaccinia described by Green, Anderson, and Smadel (1942) appeared as a central rounded area of elevation when similar preparations were shadow-casted with gold by Sharp, Taylor, Hook, and Beard (1946), it was of particular interest to determine whether such central areas would be evident when the elementary bodies of fowl pox were similarly shadowed. Although not observed in all preparations studied, it will be seen in the micrograph presented in figure 5 that such central areas of elevation are readily discernible and probably represent intraparticular material (figure 8) which becomes apparent as a result of partial collapse of the particle. In addition to the central moundlike protrusions described above it will be seen from the pictorial data presented in figure 7 that numerous strands of taffylike material, apparently under tension, connect many particles with one another, and that these strands are easily distinguished from the interparticular bridge shown in figure 2. These findings are of interest in view of the observations of Sharp, Taylor, Hook, and Beard (1946) that the particles of vaccinia also appear to be coated with a sticky material which causes coherence of some of the particles.

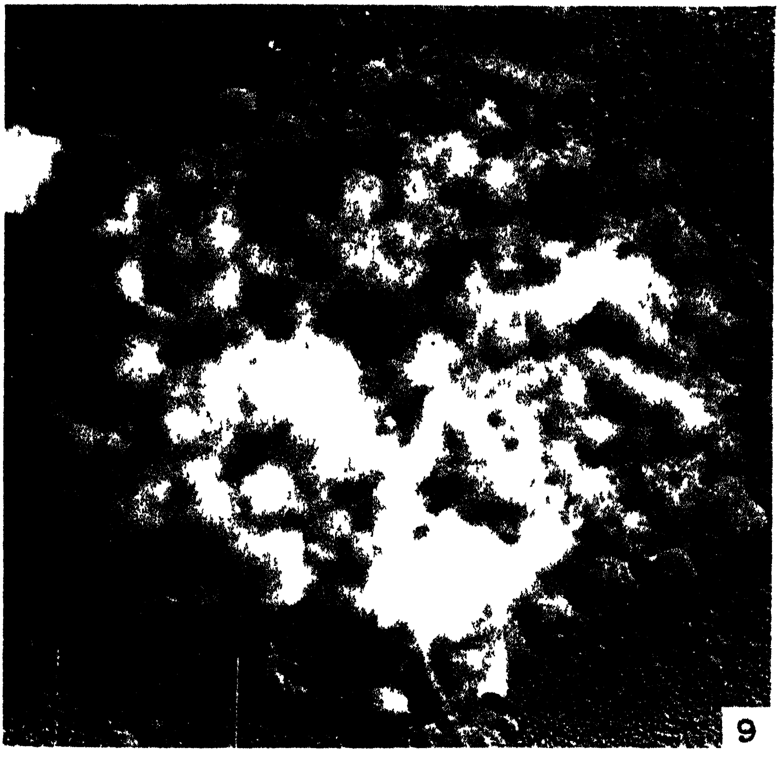


Fig. 9. Fowl Pox Virus Shadowed with 19 mg. Gold at the Angle Tangent 2/10. 2.4 × 5,900×

The sharply circumscribed, piled-up mass of elementry bodies presented in figure 9 is interpreted as representing a Bollinger or inclusion body. It will be seen that the particles are imbedded in a matrix, and that many cross-hatching strands of sticky material are present. The elementary bodies are associated with a matrix or ground substance (figures 5, 6, and 9) only when localized masses of particles are encountered during the examination of a specimen. Whether this material represents only material from the host cell or is linked with a soluble antigen such as has been described by Shepard and Wyckoff (1946) for the rickettsiae remains to be determined.

DISCUSSION

Although the elementary bodies of fowl pox are somewhat larger than those of vaccinia, the similarities between the two are striking. The particles of both appear to be approximately rectangular in shape and possess large central moundlike elevations. In addition, both seem to be coated with a sticky substance and are frequently joined to one another at their corners. That classic examples of both avian and mammalian strains should so closely resemble one another morphologically adds still another link to the chain of evidence that has bound the viruses of the pox group together. The presence of forms suggesting unequal division of elementary bodies, together with the characteristics mentioned above, supports the suggestion of Green, Anderson, and Smadel (1942) that the pox viruses have morphological characteristics that approach those of the bacteria rather than those of the plant viruses.

ACKNOWLEDGMENT

The authors gratefully acknowledge the technical assistance of Mr. John Oskay throughout these studies.

SUMMARY

The elementary bodies of fowl pox are approximately rectangular in shape, possess a central rounded area of elevation, and appear to be coated with a sticky material easily differentiated from interparticular bridges. The particles are most frequently joined to one another at their corners, and not infrequently forms are seen that suggest unequal division of the elementary bodies.

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THE EFFECT OF METABOLITES OF ESCHERICHIA COLI ON THE GROWTH OF COLI-AEROGENES BACTERIA

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For many years controversial reports have appeared in the literature to the effect that when bacteria grow in a culture medium more or less specific autoinhibitory agents are produced. Some investigators have maintained that inhibition in culture media is due primarily to a depletion of food supply.

Among investigators who believe that autoinhibitory substances are produced during bacterial growth are Garré (1887), De Giaxa (1889), Miquel (1889), Coplans (1910), Chesney (1916), McLeod and Govenlock (1921), Besredka (1923), Rideal (1923), Cornwall and Beer (1926), Grundel (1927), Weichardt (1927), Rogers (1928), Chaillot (1930), Fischer (1933), Powers (1934), Powers and Levine (1937), and Wheeler and Stuart (1937).

In contrast to these workers, Graham-Smith (1920), Barnes (1931), and Hershey and Bronfenbrenner (1937) ascribe the cessation of growth of bacteria to lack of available food material.

Layne-Claypon (1909) and Cleary, Beard, and Clifton (1935) thought inhibition to be caused by a combination of unavailability of food and the production of growth-inhibiting substances. As to the nature of the postulated growth-inhibiting products, Garré (1887), Miquel (1889), McLeod and Govenlock (1921), Rogers (1928), Powers (1934), and Powers and Levine (1937) believed such substances to be specific to a high degree. On the other hand, Weichardt (1927), Ninni and Molinari (1928), Fischer (1933), and Wheeler and Stuart believed that, while growth-inhibiting products are formed by bacteria, these products are nonspecific.

De Giaxa (1889), Miquel (1889), Eijkmann (1904), and Besredka (1923) considered the growth-inhibiting products of bacteria to be thermolabile, but Cornwall and Beer (1926), Roger (1928), and Wheeler and Stuart (1937) hold the view that such products are heat-stable. Marmorek (1902), Besredka (1923), and Wheeler and Stuart (1937) reported the growth-inhibiting products to be filterable, whereas Cornwall and Beer (1926), Grundel (1927), Rogers (1928), Powers (1934), and Powers and Levine (1937) were of the opinion that such substances were nonfilterable, i.e., the inhibiting effect is removed or markedly reduced by filtration.

The objective of the present study was to determine whether inhibition is due to autoinhibitory agents produced during bacterial metabolism.

METHODS

In the present study the technique employed was, briefly, as follows: One liter of sterile 1.0 per cent Difco proteose peptone broth (buffered with 0.1 per cent

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[1] Now with the Michigan Department of Health.

K_2HPO_4) contained in a 2-liter Erlenmeyer flask was inoculated with a culture of a member of the coli-aerogenes group and incubated at 37 C for 2 or more days. At the end of the incubation period the contents of the flask were warmed to 43 C, and equal portions of this "staled"[2] broth and a 3.0 per cent agar gel (previously warmed to 45 C) were mixed thoroughly. The mixture of broth culture and agar gel, which constituted the so-called "staled-agar-substrate," was then poured into sterile petri dishes (15 to 20 ml per dish) and allowed to harden. The outer faces of the bottoms of the petri dishes were marked off in quadrants by means of a glass-marking pencil. Each quadrant was inoculated by streaking across it a loopful of a culture of a test organism, grown for 18 to 24 hours, at 37 C in proteose peptone (1 per cent) broth. The following were employed as controls:

(1) Each organism was streaked on nutrient agar to determine "normal" vigor of growth.

(2) On smearing a loopful of a broth culture on staled media the workers sometimes observed that a film resembling very faint growth developed. To eliminate any error in respect to this phenomenon, several autoclaved broth cultures were streaked on staled media as controls.

All plates were incubated at 37 C for 48 hours, and the relative vigor of growth was recorded as follows:

0 = no growth
0.5 = slight growth
1.0 = fair growth
2.0 = moderate growth
3.0 = good growth
4.0 = heavy growth

At times interpolations between the values listed above were made. For example, 2.5 represents growth that is apparently better than that expressed by 2.0 but less than that expressed by the value 3.0.

RESULTS

Preliminary observation. Many series of experiments were made in the manner described above, but as results were fairly similar, only those for one series are presented in table 1.

It will be noted that strains which belong to the same genus as that used in producing a staled substrate were markedly inhibited by that substrate. This was especially true of strains of *Escherichia* which were completely inhibited on the two *Escherichia coli* "staled" substrates employed. Similarly, but to a lesser extent than observed with strains of *Escherichia*, strains of "*Citrobacter*," *Aerobacter aerogenes*, and *Aerobacter cloacae* were inhibited by their homologous substrates.

Since complete inhibition of *Escherichia* strains was obtained with substrates of *E. coli* (strains 100 and 115), it was felt that old broth cultures of these strains merited further study. *E. coli* (strain no. 100) was arbitrarily selected and observations made of the effect of time and temperature of incubation on the inhibiting properties of the "staled" media.

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[2] "Staled" agar consisted of equal parts 3.0 per cent Difco agar and a proteose peptone (1.0 per cent) broth culture grown at 37 C for 48 (or more) hours.

TABLE 1

Growth of members of coli-aerogenes group on staled media*

TEST STRAINS	STALED SUBSTRATES					
	E. coli (no. 100)	*E. coli* (no. 115)	"*Citrobacter*" (no. 277)	*A. aerogenes* (no. 174)	*A. aerogenes* (no. 108 A)	*A. cloacae* (no. 41)
	Relative vigor of growth, 48 hr, 37 C					
Escherichia						
107	0	0	0	1	2	0
2	0	0	0.5	1	1	1
115	0	0	0.5	1.5	1.5	2
200	0	0	1	1.5	1.5	2
100	0	0	1	1	2	2
101	0	0	1	1	1	0
133	0	0	0.5	1	1	1
"*Citrobacter*"						
79B	1.5	1 5	1	1.5	2.5	2.0
202	1	1	0	1.0	2.0	1.5
277	1	1	0	1.5	1.5	2.5
128C	1	1	0	1.5	1.5	1.0
139S	1	1	1	1.5	2.0	1.5
144S	2	1.5	1	1.5	2.5	2.0
147	1	1		1.5	1.5	1.0
A. aerogenes						
244	1.5	2.5	1.0	0.0	0.0	2.0
73	2.0	2.0	1.5	1.0	1.5	1.5
174	3.0	3.0	2.0	0.0	2.5	2.5
A1	3.0	3.0	3.0	1.0	2.5	3.0
A2	1.5	1.5	1.5	0.0	2.5	2.5
66A	1.5	1.0	1.0	0.5	0.0	2.5
108A	2.0	2.0	1.0	0.0	0.5	2.5
180D	2.0	2.5	3.0	1.5	2.0	3.0
A. cloacae						
301	1.5	1.5	1.0	0.5	1.0	1.0
251	1.5	1.0	0.5	1.0	1.0	1.0
252	1.5	1.0	0.5	1.0	1.0	0.5
41	2.0	1.5	0.5	0.5	1.0	0.5
$10A_2$	1.5	1.0	0.5	0.5	0.5	0.5
211D	1.5	1.0	0.5	0.5	0.5	0.5
$214D_1$	1.0	0.0	0.0	0.0	0.0	0.5

* Ten-day (37 C) cultures employed for staling media.

0 —no growth.
0.5—slight growth.
1.0—fair growth.
2.0—moderate growth.
3.0—good growth.
4.0—vigorous growth.

Effect of length of staling period on inhibiting properties of staled media. A series of 2-liter Erlenmeyer flasks (containing 1 liter of 1.0 per cent proteose peptone) was inoculated with *E. coli* no. 100 and incubated at 37 C for 2 to 25 days. At the end of various incubation periods a flask was removed and staled agar plate tests were made as previously described. In these tests 198 strains were used, as follows: *Escherichia*, 50 strains; *A. aerogenes*, 49 strains; *A. cloacae*, 49 strains; and "*Citrobacter*," 50 strains.

TABLE 2

Effect of age of broth culture of E. coli no. 100, employed to prepare staled agar, on growth of members of coli-aerogenes group

TEST STRAINS	AGE OF CULTURE EMPLOYED FOR STALING				
	2 Days	4 Days	10 Days	20 Days	25 Days
	Cumulative vigor of growth, 48 hr, 37 C				
Escherichia (50 strains)	2.5	0	3	0	0
A. aerogenes (49 strains)	77.5	72	53.5	51.5	30.5
A. cloacae (49 strains)	46.0	46.0	28.0	25.5	18.0
"*Citrobacter*" (50 strains)	37.0	27.5	21.0	15.5	11.5
	Per cent of strains completely inhibited				
Escherichia (50 strains)	90.0	100.0	94.0	100.0	100.0
A. aerogenes (49 strains)	0.0	4.1	4.1	6.2	2.0
A. cloacae (49 strains)	26.5	20.4	26.5	26.5	34.3
"*Citrobacter*" (50 strains)	20.0	30.0	36.0	56.0	68.0

The results are summarized in table 2, which gives the cumulative vigor of growth for each group of test organisms for a given medium. The cumulative vigor of growth for any group of organisms is a figure derived by adding the values for vigor of growth for each strain of that group. (These individual values were scored as in the preceding experiments.) For example, the data of table 2 indicate that the cumulative vigor of growth for 50 strains of *Escherichia* after 4 days' incubation of the substrate was 0. In other words, not one of the *Escherichia* strains showed visible growth upon that particular substrate. In table 2 are also given the percentages of strains completely inhibited on the various staled media.

It is evident from the results obtained that incubation of the culture substrates for as little as 48 hours was sufficient almost completely to prevent growth of *Escherichia* strains, and that the degree of inhibition of the strains of *A. aerogenes*, *A. cloacae*, and "*Citrobacter*" employed increased progressively with the age of the staled substrate. The effect of age of staling was particularly marked with the "*Citrobacter*" strains, which showed a progressive rise (from 20 per cent for the 2-day to 68 per cent for the 25-day-old staled medium) in the number of cultures completely inhibited as the age of the staling culture increased.

TABLE 3

Effect of temperature of incubation of culture used to prepare staled agar on growth of coli-aerogenes group*

TEST STRAINS	STALED SUBSTRATES							
	E. coli (no. 100)		*E. coli* (no. 115)		*A. aerogenes* A_3		*A. aerogenes* (no. 174)	
	INCUBATION TEMPERATURE							
	37 C	30 C	37 C	30 C	37 C	30 C	37 C	30 C
	Cumulative vigor of growth							
Escherichia (50 strains)	0	0	0.5	0	44.0	43.0	45.5	36.5
A. aerogenes (49 strains)	77.0	72.0	80.0	67.5	6.0	14.0	12.5	8.0
A. cloacae (49 strains)	36.0	55.5	41.5	46.5	0.5	1.5	0.5	0.5
"*Citrobacter*"[1] (50 strains)	35.5	45.0	40.5	44.5	41.0	43.5	33.5	46.5
	Percentage of strains completely inhibited†							
Escherichia (50 strains)	100	100	98	100	0	0	2	18
A. aerogenes (49 strains)	0	0	2	2	77	43	51	71
A. cloacae (49 strains)	27	14	18	20	98	94	98	98
"*Citrobacter*"[1] (50 strains)	22	8	18	8	16	4	16	8

* Twelve-day-old cultures used.

† Percentage calculated to nearest whole number.

The *Escherichia* strains were completely inhibited by a 4-day staled medium, while the cumulative vigor of growth for the *Aerobacter* strains was 72 in contrast to the low value of 27.5 for the "*Citrobacter*" strains. This relationship (i.e., the *Escherichia* strains being completely inhibited, the *Aerobacter* strains showing the least degree of inhibition, with the "*Citrobacter*" strains falling in between these) held true for each of the 5 staled media observed. It therefore appears that the "*Citrobacter*" strains are not only intermediate on the basis of the V.P.-M.R.-citrate reactions, but they are also intermediate with respect to vigor of growth on *Escherichia* staled agar media.

Effect of temperature of incubation of staled broth on inhibitory properties. The effect of temperature of incubation on the inhibitory properties of staled agar was observed, the same technique and 198 test cultures being employed as in the preceding experiments. Duplicate staled agar substrates were prepared using two sets of the following organisms (one set incubated at 37 C and the other at 30 C for 12 days): *E. coli* no. 115, *E. coli* no. 100, *A. aerogenes* A_2, and *A. aerogenes* no. 174. Each test culture was streaked on each substrate, and the plates were incubated at 37 C for 48 hours, after which the vigor of growth was recorded.

The results are summarized in table 3, where values for the cumulative vigor of growth are recorded for each group of test strains upon each substrate, and also the percentages of strains completely inhibited. The results indicate that, though the individual strains showed some variations, there was no significant difference in the inhibitory properties of staled broth produced by cultures incubated at 30 C or 37 C for 12 days.

SUMMARY AND CONCLUSIONS

Some observations were made on the production of growth-inhibitory substances by members of the coli-aerogenes group.[3]

Members of the coli-aerogenes group growing in 1.0 per cent proteose peptone broth buffered with 0.1 per cent K_2HPO_4 produced autoinhibitory agents. Those produced by *Escherichia coli* were especially effective as indicated by complete inhibition of 50 *Escherichia* strains on a staled agar made from a 48-hour (at 37 C) culture of *E. coli.*

The degree of inhibition increased with the time of incubation of the broth cultures used for preparing a staled medium.

There was no marked difference in the inhibitory agents produced by incubation of broth cultures at 37 C as compared with that at 30 C for a period of 12 days.

The inhibitory substances produced by members of the coli-aerogenes group in old broth cultures may serve as a basis for differentiation or for isolation of the various members of the coliform group from mixtures.

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[3] In a paper in preparation it will be shown that inhibition is not due to food depletion, change in pH, or the action of phage.

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A MECHANISM FOR THE DEVELOPMENT OF RESISTANCE TO STREPTOMYCIN AND PENICILLIN[1]

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Demerec (1945) and Luria (1946), in studying the resistance of staphylococci to penicillin, found that for a given inoculum (approximately 300,000,000 bacteria) there was a variation in resistance of approximately tenfold for all of their strains; e.g., if the inoculum required 0.1 unit of penicillin for complete inhibition, many of the bacteria in the inoculum would be inhibited by as little as 0.01 unit. The titer of 0.1 unit represented the resistance of a relatively small number of bacteria in the inoculum. Demerec indicated that the development of penicillin resistance resulted from the selection of the most resistant bacteria in concentrations of penicillin not completely inhibiting growth. By continued subcultures in higher penicillin concentrations resistance gradually developed through a series of small increments.

We found that, in the case of streptomycin (Klein and Kimmelman, 1946) and penicillin, if one examined only several million bacteria, there was a similar relatively small range of variation in resistance. However, by examining very large numbers of bacteria not previously exposed to streptomycin, namely, several billion, it was possible to isolate from all of six strains of the shigellae studied (standard inocula of which were inhibited by 3 to 7 units of streptomycin) variants resistant to more than 1,000 units of streptomycin. The presence of these highly resistant variants (approximately one resistant bacterium to one billion susceptible cells) was indicated to be the critical factor for the very rapid development of streptomycin resistance.

Clinically and *in vitro* (Graessle and Frost, 1946) bacteria become resistant to penicillin at a far slower rate than to streptomycin. If the few highly resistant variants are the factors determining the very rapid development of streptomycin resistance, then it follows that highly resistant variants should regularly be found present in strains showing a rapid rate of development in their streptomycin resistance. Highly resistant penicillin variants should be absent or far less frequent in strains showing a slow rate in their development of penicillin resistance.

In the present work we have tested several billion bacteria in each member of a group of strains for the presence of highly resistant variants against penicillin and streptomycin. We have found that while highly resistant variants against streptomycin were present in all the strains tested, no highly resistant variants were found against penicillin. These results were reflected by the rates at which the strains became resistant *in vitro* to the two chemotherapeutic agents.

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EXPERIMENTAL

The methods used in the assay procedures for resistant variants and the development of resistant strains are essentially similar to the procedures previously used (Klein and Kimmelman, 1946). The various penicillin and streptomycin dilutions were prepared in a final volume of 5 ml of extract broth, pH 7.3.[2]

Incidence of variants resistant to penicillin and streptomycin. In our assays very large inocula were used in order to permit the examination of many bacteria for the very few resistant variants present. The initial titers of the test strains listed in tables 1 and 2 were determined by using as our standard inoculum 0.4 ml of a 20- to 24-hour broth culture. As we have previously shown in the case of the shigellae (Klein and Kimmelman, 1946), though a given concentration of streptomycin may inhibit the growth of a single test inoculum, one cannot conclude that this specific strain cannot give rise to variants resistant to greater streptomycin concentrations. These highly resistant variants can only be found by examining very large numbers of bacteria.

TABLE 1

*Incidence of variants resistant to penicillin**

TEST BACTERIA	INITIAL TITER; UNITS OF PENICILLIN PER ML INHIBITING GROWTH	NUMBER OF POSITIVE CULTURES (RESISTANT VARIANTS) IN 100 TUBES	TEST CONC. OF PENICILLIN; UNITS PER ML	FOLD INCREASE OF TEST PENICILLIN CONCENTRATION OVER INITIAL TITER
Staphylococcus aureus no. 726	0.1	0	2.0	20
Staphylococcus aureus no. 726	0.1	0	7.5	75
Staphylococcus aureus no. 726	0.1	0	7.5	75
Staphylococcus aureus no. 4A	0.5	0	2.5	5
Staphylococcus aureus no. 5A	0.1	0	2.0	20
Staphylococcus albus no. 7	0.1	0	2.5	25
Staphylococcus albus no. 7	0.1	0	7.5	75
Staphylococcus albus no. 7	0.1	0	7.5	75
Staphylococcus aureus no. 725	0.1	0†	1.0	10
Staphylococcus aureus no. 725	0.1	0	2.5	25
Staphylococcus aureus no. 725	0.1	0	4.0	40
Staphylococcus aureus no. 725	0.1	0	7.5	75
Staphylococcus aureus no. 723	0.1	0	1.5	15
Staphylococcus aureus no. 723	0.1	0	2.5	25
Staphylococcus aureus no. 723	0.1	0	7.5	75
Streptococcus viridans	6.0	0	600	100
Streptococcus viridans	6.0	0	250	41
Staphylococcus aureus no. 1A	0.05	0	0.5	10

* Inoculum used in determining initial titer was 0.4 ml of a 20- to 24-hour broth culture (approximately 80,000,000 bacteria). Number of resistant variants in each assay was determined by seeding 0.4 ml of culture into each of 100 broth tubes containing 5 ml of indicated test concentration of penicillin.

† Growth was observed after 72 hours in one tube of this assay.

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[2] We wish to thank Dr. Chester S. Keefer, Chairman of the Committee on Chemotherapeutic Agents of the National Research Council, for our supply of streptomycin.

We tested our strains for variants resistant to penicillin and streptomycin by inoculating 100 of the standard 0.4-ml inocula into concentrations of penicillin or streptomycin many times greater than the concentration found inhibitory in the initial assays. For example, it was found that the 0.4-ml inoculum of *Staphylococcus aureus* no. 725 (table 1) was inhibited by 0.1 unit of penicillin. Four series of one hundred 0.4-ml samples were tested for variants resistant to 1, 2.5, 4, and 7.5 units of penicillin, which represented a 10-, 25-, 40-, and 75-fold increase, respectively, over the initial titer. For a given assay, if growth occurred in a single tube after 48 hours' incubation at 37 C, it indicated that one of the one hundred 0.4-ml inocula contained a bacterium resistant to the high test concentration of the chemotherapeutic agent. The results are summarized in tables 1 and 2.

Only four of the strains were tested against both of the agents, since they were the only strains sufficiently susceptible to both agents to permit an assay for resistant variants. For example, *Escherichia coli* and *Proteus vulgaris* were assayed only against streptomycin for resistant variants, since both strains grew in more than 50 units of penicillin per ml in the initial assay. As shown in tables 1 and 2, the incidence of resistant variants to the two agents differed markedly. On the assumption that growth in each tube represents the presence of one resistant variant, 57 variants resistant to high concentrations of streptomycin were found after seven assays of 100 tubes each. No highly resistant penicillin variants were found in eighteen 100-tube assays.

TABLE 2

Incidence of variants resistant to streptomycin

TEST BACTERIA	INITIAL TITER; UNITS OF STREPTOMYCIN PER ML INHIBITING GROWTH	NUMBER OF POSITIVE CULTURES (RESISTANT VARIANTS) IN 100 TUBES	TEST CONC. OF STREPTOMYCIN; UNITS PER ML	FOLD INCREASE OF TEST STREPTOMYCIN CONCENTRATION OVER INITIAL TITER
Escherichia coli	12	18	1,000	84
Proteus vulgaris	64	1	1,000	15
Staphylococcus albus no. 7	4	9	100	25
Staphylococcus albus no. 7	4	2	1,000	250
Staphylococcus aureus no. 4A	16	25	1,000	62
Staphylococcus aureus no. 5A	10	1	1,000	100
Staphylococcus aureus no. 726	16	1	1,250	80

The rate of development of resistance to penicillin and streptomycin. Since highly resistant variants were present in the streptomycin assays but not in the penicillin assays, we studied the rate at which the four test strains (table 3) became resistant *in vitro* in order to determine the correlation between the incidence of resistant variants and the rate of development of resistance. Using an inoculum of 0.1 ml of a 20- to 24-hour broth culture, 0.1 ml was subcultured from the last tube showing growth in the penicillin and streptomycin assays to higher concentrations of the respective agents. The fold increase in resistance is indicated after 3 subcultures in the case of streptomycin and after 3 and 6 subcultures in the case of penicillin. The results show that the four strains became resistant to streptomycin at a much more rapid rate than to penicillin. After only 3 subcultures all of the strains grew in 5,000 units of streptomycin per ml, the highest test concentration. The penicillin strains showed only a slight increase in resistance after 3 subcultures, and even after 6 subcultures the penicillin strains (excepting *S. aureus* no. 5a) did not reach the same level of resistance as was obtained with streptomycin

We also attempted to study the presence of highly resistant variants in sulfadiazine. However, our standard inoculum of 0.4 ml grew in a saturated solution of sodium sulfadiazine in a casein hydrolyzate medium, and even a 100-fold reduction in the size of the inoculum still gave us growth in this sulfonamide concentration (1:500). Since it was not feasible under the conditions of our assay to examine as large numbers of bacteria as had been tested with penicillin and streptomycin, no extended studies for resistant variants were done with the sulfonamides.

TABLE 3

Rate of development of resistance to penicillin and streptomycin

TEST BACTERIA	FOLD INCREASE IN RESISTANCE			
	After 3 subcultures		After 6 subcultures	
	Penicillin	Streptomycin	Penicillin	Streptomycin*
Staphylococcus albus no. 7	2	1,250	10	—
Staphylococcus aureus no 4A	10	315	100	—
Staphylococcus aureus no 5A	50	500	600	—
Staphylococcus aureus no 726	2	315	100	—

* After 3 subcultures all of the strains grew in 5,000 units of streptomycin per ml, the highest concentration tested

DISCUSSION

Our results indicate clearly that though variants showing a high degree of resistance can be found in cultures assayed against streptomycin, highly resistant variants are not found against penicillin These results indicate that the following factors are involved in the rate of development of drug resistance.

If one inoculates bacteria into partially inhibitory concentrations of, say, 4 units of streptomycin, many of the bacteria will be killed; and as the most resistant bacteria in the inoculum multiply, they will show a distribution pattern in their resistance. Since the bacteria are now growing in 4 units of streptomycin, variation of the bacteria will occur in the direction of resistance greater than 4 units, and the degree of increased resistance will be determined by the extent of variation in resistance of the new bacteria. If the bacteria give rise to cells in which the range of resistance is very great, e.g., bacteria growing in 4 units of streptomycin giving rise to variants resistant to 100 or 1,000 units of streptomycin, then the rate of development of resistance will be very rapid. This is the probable pattern of development in the case of streptomycin.

In the case of penicillin, however, there is not a broad range of penicillin-resistant bacteria, and highly resistant variants do not occur. Therefore, if bacteria are growing in partially inhibitory concentrations of, say, 0.1 unit of penicillin, the distribution of resistance in bacteria growing in this concentration will always be relatively close to 0.1 unit, and the rate of development of resistance will be slow.

Whether or not the selection of resistant variants is the only factor involved in the development of resistance is difficult to determine. Though we have previously found (Klein and Kimmelman, 1946) that the shigellae when in the resting stage show no adaptation to streptomycin after prolonged exposure, it is possible that a change in resistance can occur when streptomycin acts on susceptible dividing bacteria. The problem is difficult because, if one works with dividing cells, it would be hard to determine whether the increased resistance results from the presence of resistant variants or the interaction between susceptible dividing bacteria and streptomycin.

SUMMARY

A total of 57 variants resistant to high concentrations of streptomycin were isolated from all of six strains tested. No variants possessing a high degree of resistance to penicillin were found in a total of eight strains. The incidence of resistant variants against the two chemotherapeutic agents was correlated with the rate at which resistance developed *in vitro*.

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ANTAGONISM BY AEROBACTER STRAINS

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The literature on the antagonistic action of members of the genus *Aerobacter* is rather scanty. Winslow and Cohen (1918*a*, 1918*b*) reported that when a mixture of 46 per cent *Bacillus aerogenes* cells and 54 per cent *Bacillus coli* cells suspended in unsterilized tap water was stored 60 days, only 29 per cent of the remaining cells were *B. coli.* A slight degree of antagonism might conceivably have existed; however, Powers and Levine (1937) failed to demonstrate appreciable inhibition of *Escherichia* and intermediate" strains on "staled" media made by adding agar to 10-day broth cultures of *Aerobacter aerogenes* and *Aerobacter cloacae.* Gundel (1927) claimed *Bacterium lactis-aerogenes* to be antagonistic to *Bacillus anthracis* but stated that the strains which he used could be distinguished from colon bacilli solely on the basis of capsule formation. Ivanovics (1931) reported only one out of six *B. aerogenes* strains moderately inhibitory toward *Eberthella typhosa* in plain broth, but all six were markedly inhibitory in sodium tetrathionate broth. Wynne and Williams (1945) confirmed Ivanovics' finding of antagonism of *A. aerogenes* toward *E. typhosa* but found a much higher degree of antagonism in plain broth than in sodium tetrathionate medium. Waksman (1945) has cited Fadeeva and Tchernobaiev's 1935 report of antagonism of *A. aerogenes* toward *Bacillus pestis.*

EXPERIMENTAL

All species used in this work were repurified by single colony isolation before the investigation was initiated. The 21 species tested against the *Aerobacter* strains were from the stock culture collection of the Department of Bacteriology at the University of Texas, where their characteristics had been checked at intervals over a considerable period of time. These included *Alkaligenes faecalis, Bacillus anthracis, Bacillus cereus, Bacillus mycoides, Bacillus mycoides* 24-V (*cereus*-like variant), *Bacillus polymyxa, Bacillus subtilis* Koch-Novy, *Eberthella typhosa, Escherichia coli, Neisseria catarrhalis, Proteus vulgaris, Pseudomonas fluorescens, Salmonella aertrycke, Salmonella enteritidis, Salmonella paratyphi, Salmonella schottmuelleri, Serratia marcescens, Shigella paradysenteriae* (Flexner), *Shigella paradysenteriae* (Hiss), *Shigella sonnei,* and *Staphylococcus aureus.*

The eight *Aerobacter* strains used were *Aerobacter cloacae, A. aerogenes* L, *A. aerogenes* 29, and five strains of *A. aerogenes* numbered 1 to 5. The first three strains were obtained from the same source as the test species. Except where otherwise specified, *A. aerogenes* 29 was used throughout the investigation. The remaining five *A. aerogenes* strains (1 to 5) were isolated from stool specimens. All seven *A. aerogenes* strains were gram-negative, nonmotile short rods producing acid throughout and gas in the butt of Russell double sugar, acid and gas from lactose, sucrose, and glycerol, no liquefaction of gelatin, positive Voges-Proskauer tests, negative methyl red tests, and typical colonies on eosin methylene blue agar. The latter were pink, usually with dark reddish-purple centers, mucoid and capitate. The *A. cloacae* strain differed in being motile, not fermenting glycerol, and producing flatter colonies with a marked spreading tendency. It had apparently lost its power of liquefying gelatin, a not uncommon event according to Bergey *et al.* (1939).

All except four of the media employed were prepared from Difco dehydrated products. Eosin methylene blue agar with sucrose only was made according to the formula of the Difco *Manual* (1939), except that 10 grams per liter of sucrose were used instead of 5 grams each of sucrose and lactose. Eosin methylene blue agar with lactose only was prepared similarly. Buffered glucose broth for Voges-Proskauer and methyl red contained 5 grams per liter each of proteose peptone, K_2HPO_4, and glucose. Finally, glycerol broth was prepared from Difco nutrient broth to which was added 1 gram of Na_2HPO_4, 10 ml of Andrade's indicator, and 5 grams of glycerol per liter.

Incubations were at 37 C, except for *S. marcescens*, which was incubated at room temperature for maximum pigment production. All nutrient agar plates were incubated about 24 hours, but the eosin methylene blue media were incubated 12 to 16 hours to lessen *Aerobacter* overgrowth. Russell double sugar and carbohydrate indicator media were read at 24 hours, nutrient gelatin at 48 hours, bismuth sulfite agar at 4 days, Voges-Proskauer tests at 24 to 48 hours, and methyl red tests at 4 days.

Quantitative differentiation of the colonies of the test species from those of *A. aerogenes* 29, a necessary part of the experimental method employed, in the majority of cases afforded no special difficulty. Because of the rather slow disappearance of *E. typhosa* and *S. paradysenteriae* (Flexner) from mixed cultures, doubtful colonies on eosin methylene blue agar were picked to Russell double sugar agar for confirmation. Such a procedure was rendered necessary by the short (12 to 16 hours) incubation time used to reduce *Aerobacter* overgrowth, since an occasional slowly developing colony of the latter led to some confusion.

E. coli colonies on eosin methylene blue agar were typically purplish black and easily distinguished from colonies of *A. aerogenes* 29, but at times slow development of the latter and its production of a flat variant type led to some difficulty. The formation of colored colonies on eosin methylene blue agar is a function of acid production, as shown by Wynne, Rode, and Hayward (1942). Since the *E. coli* strain did not ferment sucrose, eosin methylene blue agar containing sucrose only (instead of sucrose and lactose) was also used, with resulting clear-cut differentiation between the colorless *Escherichia* colonies and the usual *Aerobacter* type.

Considerable difficulty was experienced at first in distinguishing colonies of the *S. sonnei* strain used, because of its rapid fermentation of lactose and the production by the *Aerobacter* strain of the flat variant mentioned above on eosin methylene blue agar. At 48 hours' incubation, however, it was found that colonies of the former species possessed a reddish-orange tinge aiding materially in their identification, as confirmed by numerous tests of doubtful colonies in

Russell double sugar agar and phenol red sucrose broth. As the *Shigella* strain did not ferment sucrose, eosin methylene blue agar containing sucrose only was also employed, with the same well-defined differences in colonies as was obtained in the case of *E. coli*. *P. vulgaris* presented difficulties in colonial differentiation quite similar to those experienced with *S. sonnei*. Since the former fermented sucrose but not lactose, eosin methylene blue agar with lactose only was employed to facilitate colony identification.

With most of the species for which nutrient agar was used, differentiation of colonies of the test organisms from those of *A. aerogenes* 29 occasioned no particular difficulty. It was found that the use of transmitted as well as reflected light was of value, particularly with *B. subtilis* Koch-Novy and *P. fluorescens*. The former produced a smooth variant type distinguished from *Aerobacter* colonies mainly by its flatter appearance and greater opacity.

Unless well isolated, *S. aureus* colonies were considerably smaller than those of *Aerobacter*. Since pigment production was too slow to be of value, gram stains of questionable colonies were made. A similar procedure was used for *B. polymyxa*, which was invariably gram-positive.

N. catarrhalis colonies were rather difficult to distinguish from those of *A. aerogenes* 29, especially since the latter regularly produced a rather bluish variant type less opaque than the usual colony. Furthermore, more than one type of *Neisseria* colonies was produced, with varying opacity. It was found that holding the plates at about arm's length between the observer's eye and a ceiling source of light allowed ready differentiation, however. Painstaking tests of colonies in phenol red glucose broth were confirmatory.

The general method used for semiquantitative determination of antagonism was similar to that of Ivanovics (1931), except that pure culture controls were included and the observations continued for a much longer period. Fulton (1937) has emphasized the importance of pure culture controls and has also cited Topley and Fielden's 1922 finding that certain organisms which could not be isolated from a young mixed culture might later become the predominant type.

A standardized loopful from a well-shaken 24-hour nutrient broth culture of the test species in question was inoculated into a tube containing about 4 ml of nutrient broth along with a similar inoculum from a like culture of the *Aerobacter* strain (or a dilution therefrom). After agitation, a carefully standardized loopful (or more) of the mixture was distributed over the surface of a differential agar plate by means of a glass elbow rod. After appropriate incubation, which varied somewhat with the medium and the test organism (usually 14 to 24 hours), the initial control ratio of colonies of test species to *Aerobacter* colonies was determined. Even though Fulton (1937) failed to confirm the contentions of Etinger-Tulcynska (1932, 1934) and Neufeld and Kuhn (1934) that antagonistic action is dependent on the relative initial numbers of two given organisms, all tubes giving initial control ratios of $> 3:1$ or $< 1:3$ were discarded, and in the great majority of cases the limits established were 2:1 and 1:2. Ordinarily 100 to 400 colonies or more were counted in determining these ratios.

After incubation of the mixed cultures for the intervals listed in table 1, each tube was thoroughly agitated and a loopful transferred to about 4 ml of sterile

TABLE 1

Ratios of test colonies to those of Aerobacter aerogenes 29 and no. of colonies on pure test culture controls

TEST SPECIES	STREAKING MEDIUM	DILUTION OF AEROBACTER	INITIAL CONTROL	24 HR		48 HR		72 HR		96 HR		120 HR		14 DAYS	
				Ratio	P. C.	Ratio	P. C.	Ratio	P. C.	Ratio	P. C.	Ratio	P. C.	Ratio	P. C.
Alkaligenes faecalis	E.M.B.	Undiluted	1–2	1–2	—	1–1	—	2–1	—	6–1	—	5–1	—	1–1	—
Bacillus anthracis	N.A.	1–100	2–1	0	15	0	—	0	—	0	—	0	—	0	—
Bacillus cereus	N.A.	1–10	1–1	1–100	20	1–30	16	1–50	50	1–4	45	1–7	—	1–1	—
Bacillus mycoides	N.A.	1–100	1–2	0	1	1–120	1	1–120	1	1–18	1	1–35	1	1–9	23
Bacillus mycoides 24-V	N.A.	1–100	1–1	0	19	0	10	1–45	—	1–60	—	1–60	—	1–18	—
Bacillus polymyxa	N.A.	1–100	1–2	0	16	0	—	0	—	0	—	0	—	0	—
Bacillus subtilis Koch-Novy	N.A.	1–10	1–1	1–100	25	1–25	40	1–45	75	1–8	—	1–9	—	1–6	—
Eberthella typhosa	E.M.B.	Undiluted	1–2	1–8	400	1–14	600	1–100	—	1–150	—	1–300	—	0	—
Escherichia coli	E.M.B.S.O. + E.M.B.	Undiluted	1–1	1–2	—	1–2	—	1–2	—	1–2	—	1–3	—	1–2	—
Neisseria catarrhalis	N.A.	Undiluted	1–1	5–1	—	5–1	—	4–1	—	5–1	—	6–1	—	2–1	—
Proteus vulgaris	E.M.B.L.O. + E.M.B.	Undiluted	1–1	1–1	—	1–2	—	1–2	—	1–2	—	1–2	—	2–1	—
Pseudomonas fluorescens	N.A.	Undiluted	2–1	2–1	—	9–1	—	35–1	—	3–1	—	8–1	—	25–1	—
Salmonella aertrycke	E.M.B.	Undiluted	1–2	1–2	—	1–3	—	1–3	400	1–4	280	1–3	270	4–1	330
Salmonella enteritidis	E.M.B.	Undiluted	1–2	1–4	—	1–5	—	1–6	750	1–10	700	1–9	500	9–1	750
Salmonella paratyphi	E.M.B.	1–10	2–1	1–30	400	1–12	—	1–20	—	0	—	0	—	0	—
Salmonella schottmuelleri	E.M.B.	Undiluted	1–1	1–1	—	1–1	—	1–1	250	1–6	200	1–10	150	1–2	210
Serratia marcescens	N.A.	Undiluted	2–1	3–1	—	1–1	260	1–2	200	1–200*	460	0*	260	0*	260
Shigella paradysenteriae (Flexner)	E.M.B.	1–10	2–1	1–25	170	1–60	—	1–300	—	0	—	0	—	0	—
Shigella paradysenteriae (Hiss)	E.M.B.	1–10	1–1	0	130	0	—	0	—	0	—	0	—	0	—
Shigella sonnei	E.M.B.S.O. + E.M.B.	Undiluted	1–1	1–2	—	1–2	—	1–2	—	1–2	210	1–7	150	1–120	120
Staphylococcus aureus	N.A.	Undiluted	1–1	0	1,000	0	—	0	—	0	—	0	—	0	—

N.A. = nutrient agar.
E.M.B. = eosin methylene blue agar.
E.M.B.S.O. = eosin methylene blue agar with sucrose only (instead of lactose and sucrose).
E.M.B.L.O. = eosin methylene blue agar with lactose only (instead of lactose and sucrose).
— = not run.
* Indicates median rather than average ratio.

0.85 per cent NaCl solution. Following agitation, an amount sufficient to give approximately 200 to 300 colonies was streaked on appropriate agar plates, and the ratio of colonies of the test species to *Aerobacter* colonies was again determined.

It should be emphasized that the ratios in table 1 are rough approximations arrived at by taking the arithmetical averages of ratios of separate experiments, except for median ratios in three instances where extreme variation made average ratios give distorted values. For example, in 13 out of 16 experiments no *S. marcescens* colonies appeared on plates streaked from 14-day-old mixtures, while the remaining three trials gave ratios of 1:3, 1:5, and 1:40. The average ratio of 1:25 obviously does not represent the typical behavior, which is more accurately given by the median ratio of 0. No attempt was made to establish cases of very slight antagonism such as Fulton's finding that *S. schottmuelleri* produced only about two-thirds as many "cell hours" in the presence of *E. coli* as was theoretically expected from pure culture controls. Ratios showing no more than threefold decreases from the initial control ratios were regarded as of no significance, and larger decreases were not interpreted as indicative of antagonistic action unless repeated pure culture controls of the test species clearly indicated such to be the case.

The pure culture controls were subjected to exactly the same treatment as the mixtures, but were run only on organisms apparently inhibited and were examined only at the intervals deemed necessary to establish the presence or absence of antagonism. Their function is best explained by examples. Colonies of *S. paradysenteriae* (Hiss) never appeared on plates streaked from mixtures; i.e., all test ratios were 0. Since pure culture controls showed an average of 125 colonies at 24 hours' incubation, marked inhibition was clearly evident. On the other hand, *B. mycoides* gave ratios of 0 at 24 hours, and 1:120 at 48 hours and 72 hours. Assuming a total of roughly 250 colonies per plate, an average of only two colonies of *B. mycoides* appeared at the last two intervals. Nevertheless, antagonism was not present, since the corresponding pure culture controls averaged only one colony. Furthermore, the mixtures yielded a 1:9 ratio at 14 days, or about 25 *B. mycoides* colonies per plate, while the pure culture controls averaged 23.

It is important in this connection to note Fulton's observation that the total growth in "cell hours" of *S. schottmuelleri* and *E. coli* in mixed cultures was 53 per cent of their total combined growth in pure culture controls. It would follow, in the absence of antagonism, that a pure culture control of a given species with a growth rate comparable to *Aerobacter* strains should yield approximately twice as many test colonies as a mixture. It is doubtful, however, on theoretical grounds of space limitations and maximum cell concentrations, that such discrepancies hold for species such as *B. mycoides* with growth rates appreciably slower than those of the *Aerobacter* strains. Ample experimental confirmation has been obtained for both conclusions.

By comparison of the data for pure culture controls with those for mixtures given in table 1, the 21 test species may be divided into four groups on the basis of the antagonistic action exerted toward them by *A. aerogenes* 29, viz.:

Group 1. Organisms so markedly inhibited that their colonies never appeared on plates streaked from incubated mixed cultures, with pure culture controls indicating that an appreciable number of colonies should have appeared in the absence of antagonism. Included are *B. polymyxa*, *B. anthracis*, *S. paradysenteriae* (Hiss), and *S. aureus*.

Group 2. Species whose colonies appeared on plates streaked from young mixed cultures, but which were not detectable or were barely detectable after a variable incubation priod. Pure culture controls showed no corresponding disappearance of or marked decrease in colonies. Included are *E. typhosa*, *S. paratyphi*, *S. paradysenteriae* (Flexner), *S. sonnei*, and *S. marcescens*.

Group 3. Organisms against which *A. aerogenes* 29 exerted a definite but temporary antagonistic action. With *B. cereus*, *B. mycoides* 24-V, and *B. subtilis* Koch-Novy this effect was manifested initially, but it did not become well defined in case of *S. enteritidis* and *S. schottmuelleri* until about 96 hours. Pure culture controls in each case demonstrated the reality of antagonism.

Group 4. Organisms not appreciably affected by the *Aerobacter* strain, insofar as could be determined by means of the method employed. Included are *A. faecalis*, *B. mycoides*, *E. coli*, *N. catarrhalis*, *P. vulgaris*, *P. fluorescens*, and *S. aertrycke*.

In table 2 are presented the results of testing seven strains of *A. aerogenes* and one strain of *A. cloacae* against *E. coli*, *S. aureus*, and *S. paratyphi*. The sources of the organisms have been given above. It is evident that some strain differences were exhibited, but the same general pattern of antagonism occurred with all *Aerobacter* organisms. None had appreciable effect against *E. coli*, but all exerted a marked action against *S. aureus*. After an initial inhibition, *S. paratyphi* effected a relative increase with all *Aerobacter* strains except *A. cloacae* to reach a maximum ratio at 48 to 96 hours' incubation, after which it disappeared from streaked plates.

The question naturally arises as to whether complete destruction occurred in cells of the species in group 1, toward which the antagonistic effect of *A. aerogenes* 29 was most pronounced. Fourteen-day-old mixed cultures of *B. anthracis* and *B. polymyxa* with the *Aerobacter* strain were heated at 60 C for 1 hour to kill vegetative cells; inoculation of 0.1 ml to nutrient broth followed. Growth occurred on incubation and each culture was identified by cellular morphology and colonial characteristics on nutrient agar as a pure culture of the test species. It may be concluded that either resistant spores were introduced in the inoculum for these two species, or else they were formed in the presence of *A. aerogenes* 29.

For nonsporulating organisms evidence was less conclusive. Smears of 14-day mixtures of *S. aureus* and the *Aerobacter* strain revealed no cells of the former, but in such old cultures staphylococci would very probably be gram-negative and quite difficult to distinguish from the coccoid *Aerobacter* cells. Furthermore, mere absence of cells of the antagonized species in smears cannot be interpreted as signifying their complete destruction.

Since no feasible experimental method suggested itself for testing this point with *S. paradysenteriae* (Hiss), the remaining organism in group 1, it was investigated with *E. typhosa*. By use of the bismuth sulfite medium of Wilson and Blair, quantitative estimations of this organism could be made fairly accurately in the presence of *A. aerogenes* 29, the large black *Eberthella* colonies with brownish halo being easily distinguished from the few smaller black colonies which the *Aerobacter* strain was able to produce on this selective medium. The accuracy of this differentiation was tested by the following procedure: 24-hour pure cultures of *E. typhosa* showed an average of 165,000,000 cells per ml by plate counts in bismuth sulfite medium, whereas mixtures with the *Aerobacter* strain yielded 40,000,000 or roughly one-fourth as many *Eberthella* cells. As shown in table 1, an average ratio of 1:8 exists between the two species in 24-hour mixtures, which should theoretically mean about 320,000,000 *Aerobacter* cells per ml—if the 40,000,000 per ml figure for *E. typhosa* is valid. Initial control ratios in table 1 reveal that *A. aerogenes* 29 produces approximately twice as many cells as *E. typhosa* in 24 hours, and 320,000,000 is roughly twice 165,000,000.

TABLE 2

Relative antagonistic powers of Aerobacter strains

TEST SPECIES	AEROBACTER STRAIN	STREAKING MEDIUM	DILUTION OF AEROBACTER	RATIO OF TEST COLONIES TO AEROBACTER COLONIES						
				Initial control	24 hours	48 hours	72 hours	96 hours	120 hours	14 days
Staphylococcus aureus	*A. aerogenes* 29	Nutrient agar	Undiluted	1-1	0	0	0	0	0	0
	A. aerogenes L	Nutrient agar	Undiluted	1-1	0	—	—	—	—	—
	A. aerogenes, 1	Nutrient agar	Undiluted	1-1	1-70	1-400	0	—	—	—
	A. aerogenes, 2	Nutrient agar	Undiluted	1-1	1-200	0	—	—	—	—
	A. aerogenes, 3	Nutrient agar	Undiluted	1-1	0	—	—	—	—	—
	A. aerogenes, 4	Nutrient agar	Undiluted	1-1	1-200	0	—	—	—	—
	A. aerogenes, 5	Nutrient agar	Undiluted	1-1	1-100	0	—	—	—	—
	A. cloacae	Nutrient agar	Undiluted	1-1	0	—	—	—	—	—
Salmonella paratyphi	*A. aerogenes* 29	E.M.B.	1-10	2-1	1-30	1-12	1-20	0	0	0
	A. aerogenes L	E.M.B.	1-4	1-1	1-16	1-8	1-10	1-30	1-40	0
	A. aerogenes, 1	E.M.B.	1-10	3-1	1-13	1-20	1-20	1-25	1-60	0
	A. aerogenes, 2	E.M.B.	1-10	2-1	1-100	1-30	1-40	1-100	1-100	0
	A. aerogenes, 3	E.M.B.	1-10	3-1	1-150	1-25	1-12	1-10	1-20	0
	A. aerogenes, 4	E.M.B.	1-10	3-1	1-15	1-12	1-30	1-40	1-25	0
	A. aerogenes, 5	E.M.B.	1-4	1-1	1-25	1-20	1-13	1-45	1-40	0
	A. cloacae	E.M.B.	1-10	5-1	1-30	1-200	0	0	—	—
Escherichia coli	*A. aerogenes* 29	E.M.B.S.O.	Undiluted	1-1	1-2	1-2	1-2	1-2	1-3	1-2
	A. aerogenes L	E.M.B.	Undiluted	1-1	1-1	1-2	1-2	1-2	1-2	1-1
	A. aerogenes, 1	E.M.B.	Undiluted	1-1	1-1	1-1	1-2	1-1	1-1	1-1
	A. aerogenes, 2	E.M.B.	Undiluted	1-1	1-3	1-2	1-2	1-2	1-3	1-2
	A. aerogenes, 3	E.M.B.	Undiluted	1-1	1-1	1-3	1-2	1-1	1-1	1-1
	A. aerogenes, 4	E.M.B.S.O.	Undiluted	1-1	1-2	1-3	1-3	1-3	1-5	1-2
	A. aerogenes, 5	E.M.B.	Undiluted	1-1	1-1	1-2	1-3	1-3	1-5	1-2
	A. cloacae	E.M.B.S.O.	Undiluted	1-2	1-3	1-3	1-2	1-3	1-4	1-2

E.M.B. = eosin methylene blue agar.
E.M.B.S.O. = eosin methylene blue agar with sucrose only.
– = not run.

Fourteen-day old pure cultures of *E. typhosa* gave a count of 64,000,000 per ml in bismuth sulfite, or about 40 per cent of the 24-hour count. If the same normal decrease in numbers be assumed for the mixed cultures, 40 per cent of 40,000,000 or about 15,000,000 *Eberthella* cells per ml, should have been present at 14 days in mixtures in the absence of antagonism. As 230,000 per ml were actually found, it may be assumed that roughly 64 out of 65 of the theoretical number were killed. Unfortunately, the antagonistic action toward *E. typhosa* was only moderate, so that these findings may not be interpreted to indicate that none of the nonsporulating bacteria affected by *Aerobacter* strains suffer complete destruction.

Filtrates of cultures of *A. aerogenes* 29 appeared completely devoid of antagonistic activity. Seventy-two-hour cultures were filtered through a Berkefeld W candle and the filtrate was added to triple strength nutrient broth in the ratio 2:1 so as to give a final concentration of nutrients at least equal to the usual broth. After incubation for sterility testing, inoculations were made of *B. anthracis*, *B. polymyxa*, *S. paradysenteriae* (Hiss), and *S. aureus*. In all cases growth was obtained which grossly equaled that in nutrient broth controls. Uninoculated controls were invariably sterile. Both 3- and 14-day-old cultures of the *Aerobacter* strain filtered through Seitz filters showed precisely the same results.

Experiments on the occurrence of antagonism in solid media gave results entirely compatible with the failure to demonstrate a filterable antibiotic "substance." Nutrient agar plates heavily seeded with *B. anthracis*, *S. paradysenteriae* (Hiss), and *S. aureus* were massively streaked down the center with *A. aerogenes* 29. On incubation none of the organisms showed visible evidence of antagonism, though it could have occurred in a very narrow zone at the surface where the *Aerobacter* growth appeared without being detectable. That this was highly probably was shown by the ease of demonstrating antagonism when living *Aerobacter* cells were in close proximity to all cells of the test species. Thirty-hour and 18-day broth cultures of *A. aerogenes* 29 were solidified with an equal quantity of a cooled solution of double strength nutrient agar, and plates prepared in this manner were streaked heavily down the center with *B. anthracis*, *B. polymyxa*, *S. paradysenteriae* (Hiss), and *S. aureus*. At 4 days' incubation, the last three species showed no visible growth, but *B. anthracis* produced a zone of growth about half as wide as that on control nutrient agar plates. Repetition of this experiment with 18-day broth cultures of *A. aerogenes* 29 heated to 60 C and 80 C for 1 hour showed no real evidence of antagonism, though the growth of the two *Bacillus* species was slightly less than on nutrient agar controls.

The extreme variability of *S. marcescens* noted above, as well as the temporary inhibition of the organisms of group 3, might be explained if resistant forms could be shown to develop from these species. Strains of *B. cereus*, *B. subtilis* Koch-Novy, and *S. enteritidis* were obtained by picking colonies developing on plates streaked from mixtures with *A. aerogenes* 29 after the initial inhibition was past and delayed growth had occurred. One strain of *S. marcescens* was obtained from a colony appearing on a plate streaked from a 14-day mixed culture that had shown no *Serratia* colonies at 120 hours, and a second strain from colonies appearing on agar prepared by solidifying a 24-hour broth culture of *A. aerogenes*

29 with 3 per cent agar-agar and streaked with the test species. Comparison of these strains with the parent cultures showed no appreciable increase in resistance, however.

A. aerogenes 29 produces a bluish, translucent, rather flat variant colony which has not been observed to revert to the usual type. Since this variant appears in varying numbers in broth cultures, any lesser degree of antagonism exhibited by it might have a bearing on the delayed growth of species in group 3. Comparison of the antagonistic effects exerted by this stable variant and those of the parent culture against *B. cereus*, *S. enteritidis*, and *S. aureus* showed no appreciable differences. Against *B. mycoides* 24-V and *B. subtilis* Koch-Novy the variant exhibited a definitely greater effect, in fact.

DISCUSSION

Though the exact mechanism of the antagonistic action of *Aerobacter* strains is unknown, it is possible that it may involve some direct action of living cells. It is interesting in this connection to note the reports of Gundel and Kliewe (1932) and Isabolinski and Sobolewa (1934) that only living cells of *E. coli* were antagonistic to *B. anthracis*. Stickelbroch (1929) also claimed that the antagonistic effect of *E. coli* toward dysentery organisms was destroyed by heating to 60 C.

The findings here reported may have a bearing on the numerous observations by many workers of the disappearance of one species of bacteria in association with another in cases where a definite antibiotic substance could not be demonstrated. Neufeld and Kuhn (1934) have used the term "direct antagonism" to describe such phenomena.

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SUMMARY

A single strain of *Aerobacter aerogenes* 29 was tested against 21 common species of bacteria and found antagonistic in varying degrees toward 14 of them.

Comparison of 8 *Aerobacter* strains against 3 test species showed only minor differences in antagonistic effects.

Whether or not complete destruction of the cells of the most strongly affected nonsporulating species occurred is uncertain. The sporeforming species most strongly affected either produced resistant spores in the presence of *A. aerogenes* 29, or these were present in the inocula.

Filtrates of *Aerobacter* cultures did not exhibit antagonism.

Heating to 60 C for 1 hour destroyed the antagonistic effect of *A. aerogenes* 29.

Attempts to demonstrate resistant forms of species only temporarily affected were unsuccessful.

A common stable variant of *A. aerogenes* 29 was found not to vary appreciably from the parent culture in its antagonistic effects.

The mechanism of the antagonistic action of *Aerobacter* strains is unknown but may involve some direct action of living cells.

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A RAPID METHOD FOR THE STAINING OF RICKETTSIA ORIENTALIS[1]

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The use of Giemsa's, Castaneda's, and Jenner's stains for the identification of *Rickettsia orientalis* in the tissues of experimentally infected laboratory animals has been described in a previous report (McLimans, Grant, and Gersh, 1944). It was found that Giemsa's stain was not entirely satisfactory for the staining of *R. orientalis* grown in the yolk sac tissues of the hen's egg. The long staining time required did not facilitate the production of large quantities of high-titered yolk sac material for use in chemotherapeutic studies (McLimans and Grant, 1945). In the absence of literature on the efficacy of the commercially available stains for the identification of the organism, exploratory investigations were done in an effort to improve the staining procedure and reduce the inconsistencies that had been encountered with the use of Giemsa's stain.

Approximately 100 slides were prepared from a Waring "blendor" ground yolk sac culture of the Karp strain of *R. orientalis* representing the fourteenth passage through hens' eggs. Stained with Giemsa's stain, sample smears were shown to contain 100 to 200 organisms per field. The slides were fixed in absolute methyl alcohol and stored for subsequent staining. A screening process for approximately 30 dyes and stains was carried out by preparing aqueous and alcoholic solutions of the stains and immersing the prepared slides in the solutions for 15 minutes. The slides were examined under the oil-immersion lens using a blue, ground-glass filter. Each preparation was graded for the following characteristics: (1) selectivity of the stain for the organism; (2) differentiation of the organism from the surrounding yolk sac tissue; (3) intensity of the staining reaction; (4) stability of the staining solution; and (5) distinguishing characteristics of the stain that enhanced or detracted from its usefulness.

It soon became apparent that methylene blue and its derivatives or combinations were the most useful stains. On the suggestion of Lt. F. Frisbee, H(W), USNR, and through the courtesy of Dr. Ward J. MacNeal, a sample of the tetrachrome stain (MacNeal, 1922) was obtained. The initial stains proved satisfactory for the identification of the organism. Repeated observations and modifications made it possible to devise a staining technique which is simple, rapid, and reliable.

DESCRIPTION OF THE TECHNIQUE AND RESULTS

The staining procedure is carried out in Coplin jars. A stock solution of the tetrachrome stain is prepared according to the directions of the manufacturer.

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[1] The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or the endorsement of the Navy Department.

The working solution is unbuffered; the optimum pH is 6.5 to 7.0. The directions for staining are as follows: (1) Prepare smears from infected yolk sacs in the usual manner and allow to air-dry. (2) Fix and kill the preparations in absolute methyl alcohol for 3 to 5 minutes. (3) Prepare the working solution of the stain by adding 4 to 5 ml of the stock solution to 75 ml of distilled water. (4) Remove the slides from the fixative and place immediately in the stain with gentle agitation. Stain for 15 to 20 minutes. (5) Clear the preparations by dipping into absolute acetone until the stain no longer dissolves from the smear. (6) Blot dry and examine under the oil-immersion lens using a blue, ground-glass filter.

Slides prepared in this manner show consistent and uniform staining. The color is retained without fading for at least 12 months. The background of the slide is a pinkish hue; the rickettsiae are stained a characteristic dull blue; erythrocytes and leucocytes are differentially stained. Bacterial contaminants are readily identified by their intense blue color and relatively large size.

CONCLUSIONS

The advantages of the tetrachrome technique for the staining of *Rickettsia orientalis* in yolk sac smears are as follows: (1) The stain is readily available either in powder or liquid form from commerical companies and remains stable over a long period of time. (2) The fixing procedure reduces to a minimum the chance for accidental infection from the slides. (3) The short staining time enables the laboratory to accommodate approximately three times as many slides daily as could be handled using Giemsa's stain. (4) The simplicity and reliability of the procedure makes it possible for inexperienced personnel to obtain good results with the staining of yolk sac cultures of *R. orientalis*. There are disadvantages in the tetrachrome technique: (1) The stain is not so intense as Giemsa's and does not lend itself to morphological studies or photomicrographs. (2) The technique does not produce consistent results in the staining of *R. orientalis* in animal tissues such as spleen and liver smears from mice and guinea pigs.

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OBSERVATIONS ON THE IN VIVO AND IN VITRO DEVELOPMENT OF BACTERIAL RESISTANCE TO STREPTOMYCIN

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The problem of acquired resistance of microorganisms is of importance in the clinical use of the sulfonamides, but present knowledge indicates it is of lesser importance with penicillin therapy. Early work (Waksman *et al.*, 1945) with streptomycin indicated that it might prove even more unfavorable than the sulfonamides in this respect, and recent work has confirmed these early indications. Persistence of infection after streptomycin therapy has been reported by Herrell and Nichols (1945) and Reimann *et al.* (1945). Buggs and coworkers (1946) noted the appearance of resistant strains after streptomycin therapy, and Miller and Bohnhoff (1946) have produced rapidly increasing resistance *in vitro* by culturing organisms on agar containing streptomycin. The latter authors showed that repeated subcultivation on agar containing streptomycin induced resistance of both gonococci and meningococci so rapidly that only 4 to 6 daily transfers to a medium containing streptomycin were necessary to permit abundant growth in the presence of 75,00 micrograms of streptomycin per ml. *In vivo* experiments confirmed the *in vitro* resistance, since the streptomycin-resistant strains of meningococci could not be controlled by the largest doses of streptomycin tolerated by animals. Finland *et al.* (1946) has recently reported that gram-negative organisms causing urinary tract infections develop a high degree of resistance to streptomycin during treatment. In a series of 12 cases of infection with various gram-negative organisms treated with streptomycin, 8 failed to show any beneficial effects. Five of these organisms on isolation were found to have acquired more than a 4,000-fold resistance to the drug following a 48-hour treatment schedule.

In an experiment designed to increase the *in vitro* resistance of a variety of organisms to streptomycin and streptothricin, it was found that some developed resistance rapidly, while others were not materially changed in their sensitivity to these drugs. Fourteen organisms from a stock culture collection were subjected to various amounts of the two antibiotics in broth. The organisms studied included gram-negative and gram-positive rods and cocci. The following method was utilized for increasing the resistance of the organisms under test:

Each organism was subcultured from a nutrient agar slant to standard methods broth, pH 7.8, and incubated overnight. With stock solutions of streptomycin and streptothricin, which were prepared by diluting weighed aliquots of these drugs in sterile 1 per cent phosphate buffer (pH 6.0), twofold serial dilutions of each antibiotic were prepared daily. The dilution series usually consisted of ten 100 × 13 mm test tubes, each containing 0.5 ml of the antibiotic dilution. To each tube was added 1.5 ml of a 1:100 dilution in broth of the 18- to 24-hour broth culture prepared above, and all tubes were incubated in a water bath at 37 C for 24 hours. The last tube showing inhibition of the organism in the dilution series indicated the initial sensitivity of the strain in micrograms of the antibiotic.

These initial sensitivities are given in tables 1 and 2. To increase the resistance of the strain to the particular antibiotic, the procedure described above was repeated, utilizing the second tube showing growth in the dilution series for preparing the 1:100 broth inoculum. Thus 0.25 ml of the mixture of the antibiotic and organisms in the second tube showing growth were added to 24.75 ml of standard methods broth (pH 7.8) and mixed, and 1.5 ml of the resulting suspension were added to each tube of a freshly prepared dilution series of the antibiotic under test. Successive transfers were not always performed on successive days.

TABLE 1

Induced resistance to streptomycin

ORGANISM	SENSITIVITY µG/ML		NUMBER TRANSFERS	INCREASE IN RESISTANCE
	Original*	Final*		
E. typhosa	0.18	>2,500	12	>1,388
E. typhosa	0.011	>2,500	14	>226,000
E. coli	0.007	3	16	428
E. coli	0.37	>12	3	>32
E. coli	0.007	0.7	8	100
S. pullorum	0.75	>250	10	>333
S. marcescens	0.15	>12	4	>80
K. pneumonia	0.015	>50	11	>3,333
S. faecalis	1.5	>2,500	8	>1,666
B. subtilis	0.011	0.37	9	33
B. circulans	0.007	1.5	7	214
B. mycoides	0.03	1.5	10	50
S. aureus	0.03	2,500	11	83,300
S. aureus	0.15	>250	3	>1,666

* Smallest amount necessary to inhibit growth.

Although all 14 organisms are relatively streptomycin-sensitive, there was a marked variation between organisms in their original sensitivity to streptomycin (table 1) ranging from 0.007 µg per ml for *Escherichia coli* and *Bacillus circulans* to 1.5 µg per ml for *Streptococcus faecalis*, a 200-fold difference. Where several strains of the same species were tested, marked differences in sensitivity were also noted. For the two strains of *Eberthella typhosa* there was an 18-fold difference; for *E. coli*, a 53-fold difference, and for *Staphylococcus aureus* a 5-fold difference. One strain of *S. aureus* became resistant quite rapidly, requiring only three transfers for an increased resistance of more than a 1,000-fold. One of the strains of *E. typhosa* exhibited a fairly rapid and extremely high increase in resistance; 14 transfers resulted in an increased resistance of 226,000-fold. There appeared to be no uniform pattern in the increase of resistance of the strains studied. An extended interval between successive transfers sometimes resulted in a loss of resistance and at other times effected an increased resistance. A sudden increase followed by a loss also was observed on successive days.

The induced resistance of the 14 organisms to streptothricin is given in table 2. When the sensitivities of the original cultures (column 1) are compared with the final sensitivities, it will be noted that there is a tremendous variation in response between the different species and strains to streptothricin. The range in concentrations of this antibiotic which caused inhibition of the original cultures of the organisms was somewhat greater than that demonstrated with streptomycin (0.0015 μg per ml for a strain of *E. coli* to 1.5 μg per ml for *S. faecalis*, a difference of 1,000-fold). Contrary to the results obtained with streptomycin, it was observed that a number of these organisms acquired a high degree of resistance to streptothricin, which subsequently was lost, and this high degree of resistance was not obtained subsequently on successive transfers.

TABLE 2

Induced resistance to streptothricin

ORGANISM	SENSITIVITY μG/ML		NUMBER TRANSFERS	INCREASE IN RESISTANCE
	Original*	Final*		
E. typhosa	0.022	1.5	22	68
E. typhosa	0.006	50	11	8,333
E. coli	0.0015	0.37	9	246
E. coli	0.15	0.37	3	2
E. coli	0.007	0.7	4	100
S. pullorum	0.045	0.7	8	15
S. marcescens	0.15	50	13	333
K. pneumonia	0.007	12.5	9	1,800
S. faecalis	1.5	12.5	13	8
B. subtilis	0.006	12	10	2,000
B. circulans	0.015	12	8	800
B. mycoides	1.5	250	14	166
S. aureus	0.03	250	14	8,333
S. aureus	0.03	12	3	400

* Smallest amount necessary to inhibit growth

In general, resistance to streptomycin was more readily acquired than resistance to streptothricin, and in most cases to a far greater degree. It is of interest that although its resistance to streptothricin was difficult to induce, *E. typhosa* readily acquired resistance to streptomycin. On the other hand, the resistance of *Bacillus subtilis* to streptothricin increased 2,000-fold in 10 transfers but only 33-fold following 9 transfers in the presence of streptomycin.

Miller and Bohnhoff (1946) reported that artificially induced resistance to streptomycin did not alter the morphological appearance of the organisms studied by them, in contrast to the marked changes in shape and physical behavior caused by penicillin. Contrary to their observations, some of the organisms employed in these studies showed changes in morphology after cultivation in broth containing streptomycin. Both strains of *E. typhosa* showed marked changes in morphology consisting of swelling, elongation of the cell, and formation of filaments. Cells showing structures resembling zygospores have been seen which are similar to the structure in *E. coli*, described by Alture-Werber *et al.* (1945), that had been isolated from the urine of a patient receiving penicillin therapy. The bizarre forms of the organisms appeared fairly early after culture in broth containing streptomycin, and persisted during subsequent transfers. A strain of pneumococcus type 1, not included in this study, likewise showed changes in morphology consisting of swelling and elongation of the cell after successive transfers in broth containing streptomycin. It should be pointed out that Miller and Bohnhoff (1946) employed a solid culture medium in their studies.

THE EFFECT OF MEDIA CONSTITUENTS ON THE ACTIVITY OF STREPTOMYCIN

Waksman and coworkers (1944) early reported the detrimental effects of an acid pH, glucose, sodium chloride, and phosphate on the activity of streptomycin. Abraham and Duthie (1946), investigating the effect of the pH on the activity of streptomycin, found that an increase in the acidity of the medium decreased the antibacterial activity of the base, streptomycin. They state that the most likely explanation is that the ionized forms of the basic drug compete with hydrogen ions for position on the cell surface. Wallace and his coworkers (1945) reported the effect of culture media on the mode of action of streptomycin and concluded that it was more difficult to inhibit bacteria on a good medium containing streptomycin than on a poor medium. The broth employed in the present investigation of induced resistance would be classed as poor. However, in preparing one test for these studies, an enriched broth was inadvertently employed, and the resulting sensitivities differed radically from those previously established. This medium, a highly nutritive buffered yeast beef broth, is used routinely in the official penicillin and streptomycin assays for potency. It differs from the standard methods broth (in containing glucose, sodium chloride, phosphate, and yeast extract, and in pH). It was decided, therefore, to examine all possible combinations of the constituents of this medium at two pH levels with 11 organisms, on the premise that the difference in sensitivities observed with the two media might be explained either by the effect of some constituent or by differences in hydrogen ion concentration.

In preliminary tests the sensitivities of each organism were established in the nutrient broth at pH 7.0 and compared with the original sensitivities, which had been obtained with the standard methods broth at pH 7.8. Every organism was at least 140 times more sensitive to streptomycin in the less nutritive, more alkaline medium. Comparison of the sensitivities of the organisms in both media at pH 7.0 and pH 7.8 showed that at pH 7.0 every organism tested was more sensitive to streptomycin in the less nutritive medium, with the difference ranging from 33-fold for *S. aureus* to 312-fold for *B. subtilis* and *B. circulans*.

At pH 7.8 the sensitivities in the standard methods broth varied from 30 times more sensitive for *E. coli* to 140 times for one strain of *E. typhosa*. This indicated that the alkalinity of the medium was not the only factor affecting the activity of streptomycin. The presence of other ingredients in the more nutritive broth either inhibited the action of the drug or provided more optimum requirements for the growth of the organisms. The fact that a more alkaline pH enhances the activity of streptomycin has been observed clinically. Finland *et al.* (1946) observed that organisms responsible for urinary tract infections which became rapidly resistant to streptomycin therapy were isolated from urine specimens that were fairly acid. In some cases, preliminary alkalinization of the urine resulted in rapid and complete elimination of the organisms from the urine after streptomycin therapy was started.

To determine which constituent or combination of constituents in the nutrient broth caused inhibition of the streptomycin or stimulation of the organism, nutrient broth was prepared at two pH levels, 7.0 and 7.8, containing every possible combination of ingredients. All the organisms were then tested simultaneously in the various broth preparations. The addition of yeast extract to a base broth did not materially alter the sensitivities of the organisms at either hydrogen ion concentration. In spite of the fact that glucose has been said to exert a detrimental effect on the activity of streptomycin, the addition of it to the base broth did not increase the resistance of organisms to streptomycin in these studies. In fact, the sensitivities of *B. circulans* and *B. subtilis* were increased in the presence of glucose, the former at both pH levels and the latter at pH 7.8. Since all of the organisms studied produce acid from glucose, the differences in their sensitivities in the two broths cannot be attributed to this constituent of the medium. The addition of yeast extract plus glucose to the base broth at pH 7.0 exerted a marked effect on the organisms since a high degree of resistance to streptomycin on the part of every organism was demonstrated. The addition of sodium chloride or phosphate to any combination of medium ingredients materially increased the resistance of 9 of the 11 organisms studied.

To emphasize the fastidious medium requirements of streptomycin for optimum activity, the organisms were studied for sensitivity to penicillin in the two media at both pH levels. In confirmation of earlier work, it was found that the composition and the hydrogen ion concentration of these media are relatively unimportant in the testing of organisms for their sensitivity to penicillin.

In order to make comparative studies of the sensitivities of organisms to streptomycin, it can be seen from this investigation that it is imperative for all laboratories to employ a broth of uniform hydrogen ion concentration containing the same constituents. Furthermore it is imperative that in testing an organism for sensitivity to streptomycin it be tested in the same batch of broth in which its original sensitivity was determined. It would be well for laboratories to maintain a reference standard medium against which all subsequent batches of media could be tested with an organism of previously established sensitivity. Differences between batches could then be calculated and a factor employed to give the corrected sensitivity. Under these conditions the results of sensitivity studies from various laboratories could be interpreted on a sound basis.

DEVELOPMENT OF IN VIVO RESISTANCE TO STREPTOMYCIN

In the *in vivo* studies white rats were employed as the test animals. These rats consisted of two litters, evenly divided as to sex and weight, and they were placed on test the day after they were weaned. All were fed on a balanced diet to which the appropriate amount of dried streptomycin sulfate was added as desired. The rats were divided into three groups of four each (A, B, and C).

All groups were maintained on a basal diet containing no streptomycin for a period of 5 days, and the fecal flora was studied bacteriologically. Total plate counts, the number of acid and gas producers (by most probable numbers), and the number of streptomycin-resistant organisms were determined. At the end of the 5-day period group A was placed on a diet containing 10,000 µg of streptomycin per 100 g of food, group B was placed on a similar diet containing 50,000 µg of streptomycin per 100 g of food, and the rats in group C were continued on the basal diet (control group). On the fifteenth day the diets of groups A and B were reversed, and on the twenty-first day they were returned to the basal diet. Group C, which acted as a control, received nothing but the basal diet during the entire experiment. A summation of the observations on the increasing number of resistant organisms isolated under these conditions is given in table 3. For the sake of brevity the data presented concern only the highly resistant organisms which were capable of growth on media containing 10,000 µg of streptomycin per ml.

TABLE 3

Increase in resistance of organisms to streptomycin in vivo

GROUP	DAYS	µG OF STREPTOMYCIN PER 100 G OF DIET	AVERAGE NO. OF ORGANISMS PER G OF FECES RESISTANT TO 10,000 MG PER ML
A	1- 5	None	0
	6–14	10,000	545,000
	15–20	50,000	1,840,000
	21–26	None	1,411,000
B	1- 5	None	0
	6–14	50,000	950,000
	15–20	10,000	950,000
	21–26	None	810,000
C	1- 5	None	8,900
	6–14	None	168,000
	15–20	None	340,000
	21–26	None	275,000

From an inspection of table 3 it is apparent that when rats are fed on a diet containing streptomycin, there appear in a relatively short time in the feces of these animals large numbers of microorganisms which are extremely resistant to this antibiotic. These organisms are mainly the fecal streptococci. It is also evident that the normal animal (control group) shows the presence of these resistant organisms, starting with the day following weaning, although they are in much smaller numbers. As the control animal grows older, the number of the resistant organisms found in the feces increases, though irregularly. The resistant organisms found in the control group were also predominantly fecal streptococci. Since the feces of the control group show the presence of streptococci resistant to streptomycin, and both groups of rats which had streptomycin in their diet showed markedly greater numbers of these same organisms in their feces, it would appear that streptomycin had the effect of stimulating the growth of these resistant organisms. The demonstration of the stimulation of the growth of an organism (hormesis) by streptomycin was previously reported (Welch *et al.*, 1946), and it was shown that certain doses of streptomycin increased the fatality rate of white mice injected with *E. typhosa.* The stimulation phenomenon may be of considerable importance in the treatment of diseases due to both streptomycin-sensitive and streptomycin-resistant organisms. Recently Finland *et al.* (1946), in treating a case of bronchiectasis in which Friedlander's bacillus was the predominating organism, found that, although the Friedlander bacillus disappeared from the sputum, following treatment with streptomycin, it was replaced by a highly resistant strain of *Hemophilus influenzae.* The latter organism had a resistance of 5,000 μg per ml. It has also been pointed out by Keefer *et al.* (1946) that one of the possible causes of streptomycin failure is that the sensitive or susceptible organisms are eliminated or decreased in numbers, and that the nonsensitive organisms multiply and invade tissues.

SUMMARY

Different species of bacteria and different strains of the same species vary greatly in their *in vitro* resistance to streptomycin and streptothricin.

Resistance of organisms to streptomycin was more readily induced than to streptothricin, and to a greater degree.

Streptomycin-induced resistance of *Eberthella typhosa* is accompanied by marked changes in morphology.

In determining the sensitivity of microorganisms to streptomycin it is important to control the composition of the medium used.

Oral administration of streptomycin to rats results in a marked increase in the numbers of streptomycin-resistant organisms.

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STUDIES ON HEMOLYTIC STREPTOCOCCI

IX. Differentiation of Species in Streptococci of Group A

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In a series of papers, the writer (1941, 1943, 1946*a*) reported the results of an incomplete study of the immunologic behavior of hemolytic streptococci of group A. Fifty-six strains representing 20 agglutinative types were examined by means of passive protective tests in mice; 11 protective antiserums were employed, each prepared against a strain representing one of 11 agglutinative types.[1]

Although the study is incomplete, correlations may now be discerned between agglutinative type, fermentative group, and immunologic behavior.

FERMENTATIVE REACTIONS

In our collection there were 326 strains of streptococci of group A, from human pathologic sources, on which agglutinative type and fermentative reactions were determined. All strains produced acid from trehalose; none produced acid from sorbitol; they differed in the production of acid from lactose, salicin, and mannitol, as shown in table 1, in which subgroups I to IV are arranged according to Brown and Schaub (1944), who omitted subgroup V.

DIFFERENTIATION OF SPECIES

Table 2 presents data on the correlation between agglutinative types and fermentative subgroups. It may be noted that the characteristics of nonfermentation of salicin and agglutination in serum of type 10 give a clear-cut distinction between the strains of *Streptococcus scarlatinae* and the strains of other fermentative groups. This species was described in the fourth paper of this series (1937), and its pathogenic properties and geographic distribution were discussed in a recent publication (1946*b*). It may be added that streptococci of type 10 capable of fermenting salicin (represented by 2 strains in table 2) have been found occasionally in this country in recent years. They are not predominantly associated with scarlet fever, as are the strains of typical *S. scarlatinae*.

Also the strains of type 3 are clearly distinguishable from the strains of other types. The data thus far obtained (Evans, 1946*a*) have indicated that the immunologic behavior of the strains of type 3 is entirely unrelated to that of any other type. In strains of type 3 the degree of virulence for mice is high and persistent throughout years of artificial cultivation, whereas that of the strains

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[1] The antiserums were prepared by injecting heat-killed bacterial cells into rabbits. The potency of the antiserums was determined by injection into mice which were inoculated the following day with living culture.

TABLE 1

Production of acid by streptococci of group A

FERMENTATIVE SUBGROUP	PRODUCTION OF ACID FROM			TOTAL NUMBER OF STRAINS OF SUBGROUP
	Lactose	Mannitol	Salicin	
I	+	−	+	222
II	+	+	+	24
III	+	−	−	31
IV	−	+	+	18
V	−	−	+	31

TABLE 2

Correlation between agglutinative types and fermentative subgroups in 326 strains of group A

AGGLUTINATIVE TYPE	FERMENTATIVE SUBGROUP					NUMBER OF STRAINS OF THE TYPE	DESIGNATION
	I	II	III	IV	V		
10	2		31			33	*S. scarlatinae*
3	21					21	*S. potens* n.sp.
6		15		9		24	Lactose-deficient group
14	9	1			7	17	
15					2	2	
17	11	3		9	1	24	
18					13	13	
19	16				2	18	
23	19	4			3	26	
11	17					17	Epidemicus group
12	8					8	
13	16				2	18	
27	15					15	
1	31					31	Pyogenes group
2	10					10	
4	11					11	
5	6					6	
8	4					4	
9	7					7	
22	2					2	
24	1					1	
25	3	1				4	
26	2					2	
28	2					2	
29	2					2	
30	7				1	8	

of the pyogenes group is comparatively low and unstable. Fuller and Maxted (1939) found that under the conditions of their tests, the strains of type 3 were sharply differentiated from those of other types of group A by their failure to produce peroxide. Long (1941) confirmed their observation and he demonstrated that strains of type 3 are very sensitive to the bacteriostatic action of sulfanilamide.

The specific name *potens*, referring to the strong virulence for mice, is proposed for strains of type 3, further characterized by failure to produce peroxide. On account of the peculiar characteristics of *Streptococcus potens* n. sp., conclusions as to the behavior of strains of other species of group A should not be drawn from tests in which strains of this species are the subject of experimentation.

THE LACTOSE-DEFICIENT GROUP

It may be observed (table 2) that with few exceptions the lactose-negative and the mannitol-positive strains (fermentative subgroups II, IV, and V) fell into types 6, 14, 15, 17, 18, 19, and 23. Many strains of fermentative subgroup I also fell into these types. On the other hand, few strains of fermentative subgroups II, IV, and V fell into other than the mentioned types.

In a previous publication (1941) the writer applied the term "lactose-deficient" to strains which, when first tested, failed to produce acid from lactose. Later, under the conditions of maintenance in our laboratory some of them acquired the ability to ferment lactose. Likewise, in our experience the ability to ferment mannitol was found to be unstable in types 17 and 23. On the contrary, all strains of type 6 were mannitol-positive when first examined, and none was observed to lose that character. Of the 49 lactose-negative strains (subgroups IV and V) in our collection, 46 fell into the seven types 6, 14, 15, 17, 18, 19, and 23. Of the 42 mannitol-positive strains (subgroups II and IV), 41 fell into the four types 6, 14, 17, and 23 (Keogh and Simmons of Australia, 1940, found that their mannitol-positive strains fell into Griffith's types 6, 17, and 23; Griffith, 1935). Eighteen of our strains belonging to types 6 and 17 fermented mannitol but failed to ferment lactose (subgroup IV).

Since all mannitol-positive strains fell into types which include lactose-negative strains, the group previously termed "lactose-deficient" includes the types which contain strains with either one or both of these characteristics.

In our experience, whenever a change occurred in the ability of a strain to produce acid from lactose or mannitol, it was always to acquire the fermentative properties of subgroup I. Lactose-negative strains became lactose-positive; mannitol-positive strains became mannitol-negative. No instance was observed of a strain of the types included in the pyogenes and epidemicus groups losing the ability to produce acid from lactose, or acquiring the ability to produce acid from mannitol.

The acquisition of the ability to ferment lactose by a strain of fermentative subgroup V would change its classification to subgroup I; the loss of ability to ferment mannitol in a strain of fermentative subgroup II would change its classification to subgroup I. The shifting of fermentative characters toward those of subgroup I would provide an explanation for the occurrence of strains of this subgroup in the same agglutinative types with lactose-negative and mannitol-positive strains of subgroups II, IV, and V. In the present state of knowledge concerning hemolytic streptococci of group A, this shifting of fermentative characters adds to the difficulties of classification.

Since some fermentative reactions are unstable and, as shown in a previous publication (Evans, 1946*d*), agglutinative reactions are unstable, these characters are of limited value for classification purposes. Immunologic behavior, a character which appears to be stable (Evans, 1946*a*, 1946*d*), is more reliable.

An explanation is in order for the apparent inconsistency in table 2 of placing in the lactose-deficient group type 19, with only two lactose-negative strains, whereas type 13, which also has two lactose-negative strains, was not placed in that group. The reason is that the immunologic behavior of the strains of type 19 was found to resemble that of strains of the lactose-deficient group, whereas the immunologic behavior of strains of type 13 did not resemble that of strains of the lactose-deficient group. For similar reasons, types 25 and 30 were placed in the pyogenes group, although each contained one strain with a fermentative character which would have placed it in the lactose-deficient group.

The position of some strains is not clear. For example, confusion concerning the position of type 23, which includes a few lactose-negative and also a few mannitol-positive strains, arises because, although neither of the two strains of this type which were studied for immunologic behavior reacted with any heterologous antiserum, nevertheless a protective antiserum prepared against one of them (no. 1072) reacted with many strains not included in the lactose-deficient group, as well as with all strains included in that group (Evans, 1946*a*).

It was reported previously by Evans (1941, 1943, 1946*a*) that strains of the lactose-deficient group are characterized by a broad antigenic structure. Certain immunologic data taken from an earlier publication (Evans, 1946*a*) which show relationships between the strains of this group are arranged in table 3 to show that strains of different fermentative groups may behave alike in immunologic tests. All 7 strains included in the table reacted with the same 7 of the 11 protective antiserums, with a single discrepancy in each of two strains. The table includes 2 strains of fermentative subgroup II, 2 of subgroup IV, and 3 of subgroup V. The mannitol-positive strains of subgroups II and IV show the same immunologic behavior as the mannitol-negative strains of subgroup V. Likewise the lactose-positive strains of subgroup II show the same immunologic behavior as the lactose-negative strains of subgroups IV and V. These data show that when a single unknown strain is to be identified, fermentation or nonfermentation of lactose and mannitol are unreliable characters for the differentiation of species.

In the earlier study (1946*a*) it was found that other strains of the lactose-deficient group, not included in table 3, reacted with fewer antiserums, but all were characterized by ability to react with one or more of the antiserums 658 of type 14, 654 of type 15, 1,268 of type 18, and 660 of type 19. On the other hand strains of types 1, 2, 3, 9, 11, 12, 13, 25, 27, 28, and 30 of fermentative subgroup I did not react with any of the four mentioned protective antiserums, with a single discrepancy in the case of 2 strains.

This difference between the immunologic behavior of strains of the lactose-deficient group, on the one hand, and strains of the epidemicus and pyogenes groups, on the other hand, appears to offer a sound basis for the differentiation of species.

In 1916 Holman gave the name *Streptococcus infrequens* to streptococci capable of fermenting mannitol as well as lactose and salicin. Apparently no strain of his *S. infrequens* is now available for study. Since his description would apply to the majority of strains of group D and to some strains of group E, as well as to some strains of group A, there appears to be no valid reason for applying the specific name *infrequens* to the mannitol-positive strains of group A.

There is better reason for the acceptance of the name *alactosus* for the species loosely defined in this paper as the lactose-deficient group. This specific name was given by Brown (1919) to lactose-negative, mannitol-positive strains which had been isolated in 1913 by Smith and Brown (1915) as the causal organism in an outbreak of sore throat (several of their strains are still available for study). In high degree of virulence for mice the majority of strains of the lactose-deficient group resemble the strains of *S. potens* n. sp. and differ from the strains of the pyogenes and epidemicus groups. Because the lines of demarcation of the lactose-deficient group are not yet clear, further studies should be made before the species is defined.

TABLE 3

The immunologic behavior[*] and the agglutinative and fermentative reactions of strains of the lactose-deficient group

STRAIN USED FOR INFECTION OF MICE			ANTISERUM PREPARED AGAINST STRAIN										
No.	Type	Fermentative group	732 (Type 3)	1527 (Type 1)	775 (Type 13)	638 (Type 9)	778 (Type ?)	1072 (Type 23)	1475 (Type 6)	654 (Type 15)	1268 (Type 18)	660 (Type 19)	658 (Type 14)
660	19	V	−[†]	−	−	−	+	+	+	+	−	+	±
1475	6	IV	−	−	−	−	+	+	+	+	−	+	+
1389	6	IV	0[‡]	0	0	0	0	+	+	+	+	+	±
996	6	II	−	−	−	−	+	+	+	+	+	+	+
1085	6	II	−	−	−	0	+	+	+	+	+	+	+
1268	18	V	−	−	−	−	±	+	+	+	+	+	+
1135	18	V	−	−	−	0	±	+	+	±	+	+	+

[*] These data on protective reactions were taken from an earlier publication (Evans, 1946*a*).

[†] − indicates no protection; + indicates protection; ± indicates evidence of slight protection.

[‡] 0 indicates not tested.

THE EPIDEMICUS AND PYOGENES GROUPS

It may be noted in table 2 that there is no difference between the fermentative reactions of the strains included in the epidemicus and pyogenes groups. The reasons for recognizing a distinction between them are not yet entirely clear, although the bacteriologists of several decades ago regarded the characteristics of encapsulated cells and watery colonies of the streptococci associated with outbreaks of septic sore throat as distinctive of a species, *Streptococcus epidemicus*, first described by Davis and Rosenow (1912).

The writer has found further evidence of distinguishing characters in the strains of types frequently associated with outbreaks of septic sore throat. It was shown (Evans, 1940) that there is some correlation between resistance of streptococci of group A to bacteriophage C/594 and association with outbreaks of septic sore throat. More recently (1946*c*) observation was made of the tendency of the agglutinative types 11, 12, 13, and 27, included in the epidemicus group, to produce bovine mastitis associated with epidemic sore throat. Furthermore, an immunologic relationship has been found between the strains of these types (table 2; Evans, 1946*a*).

Probably some of the types which in table 2 are placed in the pyogenes group will be found to belong more properly in the epidemicus group. For example, a study of the immunologic behavior of single strains of types 2, 9, and 25, respectively (Evans, 1946*a*), suggests that one or more of these types may be found to belong more correctly in the epidemicus group.

Brown and Schaub (1944) suggested that mannitol-positive strains of group A may have been the parent stock from which other strains of group A were derived. The writer concurs, and further believes that there is evidence that the lactose-negative strains of group A were derived from animal strains of group C, and that further deviation from the lactose-deficient group resulted in strains with the characteristics of the pyogenes and epidemicus groups.

In the seventh and eighth papers of this series (Evans 1944*a*, 1944*b*) the sequence of relationships of *Streptococcus equi* (group C) to *Streptococcus equisimilis* (group C) to lactose-negative strains of group A was discussed. The relationship between *S. equi* and *S. equisimilis* was shown by the demonstration of protection of mice against strains of *S. equisimilis* by antiserums prepared against strains of *S. equi;* reciprocal protection was not found in the one test that was made. A relationship between strains of *S. equisimilis* and lactose-negative strains of group A was shown by the protection of mice against lactose-negative strains of group A by antiserum prepared with a strain of *S. equisimilis.* (See table 6 of the eighth paper of this series, where all strains of group A which reacted with antiserum 790 of type 21, group C, belonged to the lactose-deficient group.) Positive reactions were obtained also in reciprocal experiments. No reaction was obtained, however, in experiments designed to show a protective relationship between strains of *S. equisimilis* and lactose-positive strains of group A. In a recent paper (Evans, 1946*a*) it was shown that antiserums prepared against the mannitol-positive, lactose-positive strain 1072 of type 23 and the mannitol-positive, lactose-negative strain 1475 of type 6, respectively (both of the lactose-deficient group), gave protection against many strains of the pyogenes and epidemicus groups, but no reaction was obtained in the reciprocal tests which have been made.

This sequence of protective relationships suggests the route of derivation of the strains of the pyogenes and epidemicus groups as follows:

$$\textit{S. equi} \rightarrow \textit{S. equisimilis} \rightarrow \text{lactose-deficient group} \rightarrow \begin{cases} \text{pyogenes group} \\ \text{epidemicus group} \end{cases}$$

Evidence that the route of derivation was not the reverse of that suggested is found in the observation, already discussed, that changes in fermentative characteristics were always in the direction of fermentative subgroup I.

Further evidence of a relationship between strains of *S. equisimilis* (group C) and lactose-negative strains of group A was observed when precipitin tests were made to determine the serologic grouping of the strains of our collection, as reported by Evans and Verder (1938). There was especial difficulty in the case of six lactose-negative strains which reacted with serums of both groups, A and C. On a repetition of the tests, with a refinement of the technique, all six strains fell into group A.

Moreover, the high degree of virulence for mice of the majority of strains of the lactose-deficient group suggests relationship with the animal strains of group C, whereas the comparatively low degree of virulence of strains of the pyogenes and epidemicus groups suggests a more remote position.

SUMMARY

A study of many strains of beta hemolytic streptococci of group A showed that correlations exist between fermentative, agglutinative, and immunologic behavior.

The information at hand permits the recognition of two well-defined species, *Streptococcus scarlatinae* and *Streptococcus potens* n. sp. The rough outlines of three other species, *Streptococcus pyogenes*, *Streptococcus epidemicus*, and *Streptococcus alactosus* are taking shape, but further study is required to determine the lines of distinction.

A typical strain of *S. potens* n. sp. belongs to Griffith's type 3; possesses a high degree of virulence for mice; belongs to an immunologic entity unrelated to other species; produces no peroxide; and is very sensitive to the bacteriostatic action of sulfanilamide.

On account of the peculiar characteristics of *S. potens* n. sp., conclusions as to the behavior of strains of other species of group A should not be drawn from tests in which strains of this species are the subject of experimentation.

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NOTES

A TYPHOIDLIKE INFECTION WITH SALMONELLA GATUNI

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The first and only culture of *Salmonella gatuni* (VI.VII;b-enz15) was typed and reported by Wilcox and Coates (J. Bact., **51**, 561). It had been isolated by Menzel from the stool of a waitress in the Canal Zone in 1945. No clinical data were available.

This is a report on the second isolation of this organism the serological and cultural type identification of which was established in the National Salmonella Center, New York City.

A 31-year-old man, who had received a full course of TAB vaccine 3 months previously, came down April 1, 1946, with chills and fever which reached 39 and 40 C. On April 14 the temperature dropped critically to 37 C, where it remained for 4 days. The patient was hospitalized on April 14. On April 19 the temperature rose again and within 2 days reached 41 C. For 1 week the temperature remained between 39 and 40 C and then gradually returned to normal within the following 8 days as the other symptoms subsided. The temperature was normal on May 5, and remained so. During the fever period in the hospital, the pulse rate oscillated between 110 and 120. A white cell count on April 18 was 7,500. The blood picture was essentially normal. No roseola nor enlarged spleen was noted. There was only slight diarrhea without slime and blood for 2 days. The general aspect of the patient, who complained of weakness, malaise, and headache, was that of a man severely ill with typhoid fever. The Widal reaction for typhoid was 1:40 on April 19, and 1:80 on April 25. Agglutinins for *Salmonella paratyphi* A and B were not present. *S. gatuni* was isolated from the stool of the patient on April 25, and the agglutination with the patient's serum against this organism was positive at 1:300.

Two conclusions can be drawn from the history of this infection with *S. gatuni*: (1) A typhoidlike syndrome can be caused by an organism other than *Salmonella typhi* and *S. paratyphi* A and B. This has been stressed time and again by many workers in this field. (2) Vaccination with TAB vaccine, even as recently as 3 months, which has been found effective against members of the A, B, and D groups of the Kauffmann-White schema, does not protect against members of the C group (VI . . . .), to which belong *S. gatuni* and some other widely spread *Salmonella* organisms such as *S. cholerae-suis*, *S. oranienburg*, *S. montevideo*, and *S. newport*.

A CELL WALL STAIN EMPLOYING A CATIONIC SURFACE-ACTIVE AGENT AS A MORDANT

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For studying the morphology of bacteria in fixed smears, Knaysi (J. Bact., **49**, 375) demonstrated the value of staining by a method showing the cell wall. Knaysi (J. Bact., **41**, 141) developed a tannin alum fuchsin method which stains differentially the cytoplasm, cell wall, and capsule. It is the purpose of this note to report another cell wall stain in which the cell wall is positively charged by treatment with a cationic surface-active agent (Dyar and Ordal: J. Bact., **51**, 149) and is then stained with an acid dye. The cytoplasm may be stained with a contrasting basic dye.

By use of the following procedure, the cell wall is stained red and the cytoplasm, blue: (1) prepare and heat-fix a smear; (2) add 3 drops M/100 (0.34 per cent) cetyl pyridinium chloride; (3) add 1 drop saturated, aqueous Congo red, mixing on the slide; (4) wash; (5) counterstain for a few seconds with methylene blue; (6) wash; and (7) examine either in a drop of water under a cover slip or in oil. Side illumination is often helpful in making observations.

Species of *Bacillus*, *Micrococcus*, and *Escherichia* and also of *Saccharomyces* and *Schizosaccharomyces* have been stained in this way. The relatively thick cell wall of yeasts is seen to be stained light red and to be accentuated by a red precipitate of dye-surface-active agent on its surface. Presumably the same thing occurs with bacteria and accounts for the clarity of the stained cell wall, which has been shown by electron photomicrographs to be actually very thin. This is further indicated by the fact that occasional bacterial cells, dislodged in the staining procedure, leave on the slide an outline of stain corresponding in shape to the stained cell wall.

When bacteria, stained by this method, are examined in water under a cover slip, the cytoplasm is sometimes seen to be shrunk away from the cell wall. When such cells of *Bacillus cereus* are examined dry in oil, it is frequently seen that the cell wall has contracted, now adhering closely to the shrunken cytoplasm at the sides, though not at the ends. It appears that stained cell walls maintain the rigid form of the living cells when wet but tend to shrink or collapse when dry.

Although the dye-surface-active agent combination does not stain the cytoplasm, this is not considered evidence that the cationic surface-active agent has not penetrated the cell membranes, a possibility discussed by Hotchkiss (Ann. N. Y. Acad. Sci., **46**, 479). Actually, the cytoplasm of vegetative cells suspended in a droplet of cetyl pyridinium chloride stains black with Sudan black B, indicating that the surface-active agent has penetrated into the cell and that its hydrocarbon portion is stained by the fat dye.

A TYPE d STRAIN OF HEMOPHILUS INFLUENZAE PREVIOUSLY DESIGNATED PROVISIONALLY AS TYPE d_2 AND TYPE g

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In 1937 Dr. J. Mulder sent me a type-specific culture of *Hemophilus influenzae* (D99) which he had obtained from Dr. Van Lookeren Campagne. With the supernatant of a broth culture, a ring reaction was obtained in type d antiserum; none was obtained in types a, b, c, e, or f antisera. The precipitation, however, was not heavy, and at the time it was thought that the strain might be of another type related to d. I tentatively designated it as d_2 (Van Lookeren Campagne: Maandsch. Kindergeneeskunde, **7**, 43). On the basis of my report to him, Dr. Mulder provisionally called it type g (Mulder: J. Path. Bact., **48**, 175).

Four years later, I received a strain, Adamson, from Dr. Colin M. McLeod, labeled type d. This time a heavy precipitin reaction was obtained in the same serum that had been used in the test of the strain from Dr. Mulder.

Recently a comparison of the two strains which had been kept in the dried state was made. Antisera were prepared against cultures of the respective strains. In each case, with capsular swelling reaction, bacteria of the heterologous strain gave a positive reaction to the same titer as did those of the homologous strain, and each serum absorbed with bacteria of either the homologous or the heterologous strain lost its capacity to induce a swelling of the capsule of the bacteria of either strain. Furthermore, the antiserum produced in 1931 against the first strain, no. 218, which was designated as type d, and which had been used for the precipitin reactions on receipt of D99 and Adamson (author's numbers are 522 and 611, respectively), induced a swelling of the capsule of the bacteria of each strain, and when absorbed with the bacteria of either strain no reaction could be obtained with either one.

It, therefore, appears that the type of the strain D99 which was provisionally designated both as d_2 and g is d. This identification will permit the use of g for the designation of a type, different from a to f, that may be isolated subsequently.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

NEW JERSEY BRANCH: THE THEOBALD SMITH SOCIETY

Princeton University, Princeton, New Jersey, January 9, 1947

Quantitative Measurement of Growth of Mycobacterium tuberculosis: Effect of Streptomycin. *Dorothy G. Smith*, New Jersey Agricultural Experiment Station, New Brunswick, New Jersey.

A simple, safe, and accurate method is described for the quantitative estimation of the growth of *Mycobacterium tuberculosis*. This method is based upon turbidimetric measurements using Dubos' medium for the growth of the organism. This medium has also been modified so as to permit a study of the physiology of the tubercle bacilli. This procedure also lends itself readily to the study of the effect of antibiotic agents upon the growth of *M. tuberculosis*. Data were presented to illustrate the effect of streptomycin.

Wartime Studies on Cattle Plague. *Richard E. Shope*, Rockefeller Institute for Medical Research, Princeton, New Jersey.

OHIO BRANCH

Columbus, Ohio, December 7, 1946

Some Observations on a Pleomorphic Bacillus Isolated from Different Lots of Processed Whole Milk Products. *Helen Z. Knight and Edwin H. Browne*, M & R Dietetic Laboratories, Inc., Columbus, Ohio.

An incompletely identified pleomorphic bacillus was frequently isolated from varying lots of processed whole milk products. The morphologic units of this bacillus consisted of long rods in chains, rods with central and plectoral globi, and dumbbell forms. A definite life cycle could be observed, beginning with the long rods and ending with the clumps of globi. Increasing the nitrate content of the medium resulted in the formation of filaments resembling a mycelium. On blood agar the bacillus appeared as a small plump rod, but was not hemolytic. These variations were not permanent; the bacillus reverted back to its original forms as soon as it was transferred to standard milk agar. As far as could be determined, the presence of this bacillus in the milk product had no effect upon the product.

The Action of Phenylmercuric Nitrate IV: The Ability of Sulfhydryl Compounds to Protect Against the Germicidal Action of Basic Phenylmercuric Nitrate. *G. W. Thomas and Elton S. Cook*, Institutum Divi Thomae, Cincinnati, Ohio.

The growth-inhibiting action of basic phenylmercuric nitrate on *Escherichia coli*, *Eberthella typhosa*, and *Staphylococcus aureus* can be prevented by the sulfhydryl-containing compounds cysteine, homocysteine, and glutathione, but not by cystine and methionine. This activity was demonstrated by the use of a filter paper disc method and in broth cultures.

Errors in Compounding and Testing Semisolid Products Containing Quaternary Ammonium Salts. *Robert A. Quisno and Milton J. Foter*, Department of Bacteriology, Laboratories of The Wm. S. Merrell Company, Cincinnati, Ohio.

Results were reported which showed that agar reduces the germicidal potency of certain quaternary ammonium salts. The recommended agar plate methods for the evaluation of the antiseptic properties of ointments, creams, jellies, suppositories, etc., containing quaternary ammonium salts were thus found inappropriate. A method for determining critical killing time was reported which eliminated the use of agar. The test involved the inoculation of 5 g of the preparation with 0.5 ml of the test organism and subculturing 0.4 ml of the mixture into 10 ml of a liquid neutralizing medium after various time intervals. Tests on several preparations containing quaternary ammonium salts showed them to be germicidal by the method reported.

It was also found that certain other excipients reduce the activity of quaternary ammonium salts. It is recommended that when the formulation of preparations containing this type of germicide is contemplated the compatibility should be investigated first.

Studies on the Dehydrogenases of Clostridium perfringens. *Alfred A. Tytell and Alice G. Tytell*, Department of Biological Chemistry, University of Cincinnati, College of Medicine, Cincinnati, Ohio.

Data were presented to show that the glucose dehydrogenase of washed suspensions of *Clostridium perfringens* was inactivated by extreme dilution. The critical concentration was approximately 500 to 1,000 mg (dry weight) of washed organisms per ml. The presence of inorganic phosphate is a requisite for activity. Inactive suspensions can be reactivated by various fractions of yeast and liver extracts. Among the substances shown to reverse this inactivation are adenine, cysteine, sodium pyruvate, and methionine. Adenine can be replaced by other purines such as guanine, hypoxanthine, and xanthine. Glucose dehydrogenase can be inactivated by sodium iodoacetate, and the inhibition can be reversed by the compounds mentioned above. Streptomycin inhibition can likewise be reversed.

Studies on Eremothecium ashbyii. *Helen Norris Moore, G. de Becze, and E. Schraffenberger*, Schenley Distilleries, Inc., Biochemical Research Laboratory, Lawrenceburg, Indiana.

Riboflavin synthesis by *Eremothecium ashbyii* has been studied in various liquid substrates, utilizing the shaken flask propagation method. In yeast extract glucose peptone broth containing 2 per cent solids, at the end of 72 hours 198 mg of riboflavin were produced per ml of liquid and 124 mg per ml in distillers' thin stillage diluted to 2 per cent solid content. Supplements, such as molasses, corn oil, sugars, and inorganic salts, added to the abovementioned substrates increased the riboflavin content to as high as 356 mg per ml.

The optimum temperature was found to be 26 to 30 C, and an initial pH of 5.5 to 6.5 for both rapid development of the organism and for riboflavin synthesis was needed. Lower initial pH values retarded both the growth and riboflavin production. Under favorable conditions maximum riboflavin content is reached in 72 hours. With the progress of mold growth, the pH of the substrate, due to deamination, often increases up to 9.0.

During the course of these experiments, it was observed that when the culture of *E. ashbyii* was kept at low temperatures (+4 C) for more than 7 days, its riboflavin-producing ability was greatly decreased, and when the culture was lyophilized, by standard procedures, it completely lost its ability to produce riboflavin. Cultures grown in maltose broth maintained their riboflavin-producing ability over a 6-month period when kept at room temperature.

Effect of Addition of Streptomycin to Submerged Cultures of Streptomyces griseus. *G. L. Christenson, F. J. Rudert, and Milton J. Foter*, Department of Bacteriology, Research Laboratories of The Wm. S. Merrell Company, Cincinnati, Ohio.

Streptomycin was added to shaken cultures of *Streptomyces griseus* and the peak yield of streptomycin in such cultures was increased in the majority of cases. The addition of streptomycin to aerated cultures of *S. griseus* resulted in similar increases in the amounts of streptomycin produced. The majority of isolates obtained from shaken cultures of *S. griseus* grown in streptomycin-containing medium are capable of producing more streptomycin than their purest strain.

Correlation Between Protective Capacity for White Rats and Precipitable Antibody Content of Anti-

Tularense Serums.

Lee Foshay, I. Ruchman, and Paul S. Nicholes, Department of Bacteriology, College of Medicine, University of Cincinnati, Cincinnati, Ohio.

Antitularense serums have hitherto failed to reveal protective antibody against challenge by strains of maximal virulence whenever the test animals were the mouse, guinea pig, hamster, or rabbit. Since the reaction to tularemic infection by the rat approximates more closely that of man than do those of other animals, the rat was used for serum protection experiments.

Protective antibody was readily demonstrated in 2-ml quantities of each of 4 antitularense serums by I.-P. injection of the 85- to 110-g white rat and by immediate subcutaneous challenge with 25 million viable organisms of maximal virulence, an average of 25,000 LD_{50} rat doses.

The precipitable antibody content of serums was quantitated by the Culbertson neutralization method, using a polysaccharide prepared from acetone-extracted cells of a virulent strain by the phenol extraction method of Palmer and Gerlough.

Rat mortality was the criterion by which serum protection was judged.

Prechallenge treatment with normal serums gave 95 per cent mortality and with the most potent hyperimmune serum, aged 15 months, 12.5 per cent mortality. Serums of lesser potency gave correspondingly greater mortality. Mortality rates were roughly inversely proportional to the precipitable antibody contents of serums.

The difference in clinical effectiveness between the two best serums was of the order of that shown by both rat protection and antibody content tests.

Bacteriologic Studies in Epilepsy and Schizophrenia.

Edward C. Rosenow, Bacteriologic Research, Longview Hospital, Cincinnati, Ohio.

Alpha streptococci having specific properties have been isolated consistently from the end point of growth of serial dilution cultures in glucose brain broth from the nasopharynx and apices of pulpless teeth of persons suffering from idiopathic epilepsy and schizophrenia and from the blood in 35 per cent of epileptics and 11 per cent of schizophrenics.

The streptococci from epileptics caused spasms and convulsive seizures in high incidence in mice, rabbits, and monkeys.

Intracutaneous injection of streptococcal antiserum and thermal antibody and antigen was followed immediately by erythematous reactions diagnostic, respectively, of specific antigen and antibody in skin or blood; and subcutaneous injection of thermal antibody in therapeutic amounts caused a prompt reduction in antigen, a rise in antibody as determined by intracutaneous and agglutination tests, and often concomitant clinical improvement.

Isolation of the Virus of Herpes Simplex from Five Cases of Kaposi's Varicelliform Eruption.

Isaac Ruchman, Ashton L. Welsh, and Katharine Dodd, The Children's Hospital Research Foundation and the Departments of Bacteriology, Pediatrics, and Dermatology, University of Cincinnati, College of Medicine, Cincinnati, Ohio.

Kaposi's varicelliform infection is characterized by an acute exanthematous eruption which is superimposed over an eczema of long standing. From the skin lesions of three adults and two children presenting this syndrome a filterable agent was recovered which, by histological methods and appropriate cross-immunity tests in animals, was shown to be a strain of herpes simplex virus. An increase in antibodies during convalescence was demonstrated in three patients; one fatal case in an adult had no antibodies in the acute stage. Neutralizing antibodies were present during the acute phase of the illness in the one remaining adult, but no increase in antibody titer subsequently occurred either during convalescence or during the period which followed a late recrudescence of the disease about 5 weeks later.

The Effect of Influenza Virus Infection on the Susceptibility of White Mice to Streptococcus hemolyticus.

Harold N. Carlisle and N. Paul Hudson, Department of Bacteriology, The Ohio State University, Columbus, Ohio.

A study was made of the effect of influenza virus infection on the susceptibility of white mice to *Streptococcus hemolyticus* (group C) administered by intranasal instillation. Two hundred 14- to 16-g mice were inoculated intranasally with 0.05 MLD of influenza virus, type A, an amount which regularly produced slight symptoms but no deaths. At intervals of 0, 2, 4, 8, 12, 16, 24, and 32 days later, a suspension of streptococci was titrated in mice from the virus-prepared group and in control mice. The virus-infected mice were fatally susceptible to a 100-fold higher dilution of the streptococcus suspension, 4 and 8 days after inoculation of the virus, than were the control mice. Significant differences in susceptibility were observed also 2 and 12 days after the virus inoculation. All mortally infected mice yielded streptococci in the lungs and blood; in the latter in pure culture. Additional controls indicated that a preliminary inoculation of heat-killed virus had no effect on susceptibility to the streptococcus and that a secondary inoculation of heat-killed streptococci had no demonstrable effect on the course or outcome of the virus infection.

NEW YORK CITY BRANCH

New York, January 3, 1947, Ward J. MacNeal Memorial Meeting

The Significance of, and Methods for, Maintaining Moisture in Bacteriological Culture Media. *George H. Chapman*, Clinical Research Laboratory, New York.

There is an average loss of 0.1 ml of moisture from the surface of an agar plate each day it is stored in an electric refrigerator. The resulting dry surface is detrimental to microbic growth. The loss of moisture can be greatly reduced by storing the plates in metal cylinders sold for this purpose and sealing the joint between top and bottom sections, preferably with waterproof adhesive tape or with parafilm or a rubber band. The vent hole should also be sealed. Tubes of culture media can be sealed by covering the plug or screw cap and rim with parafilm.

A Superior Culture Medium for the Enumeration and Differentiation of Coliforms. *George H. Chapman*, Clinical Research Laboratory, New York.

The medium is based on the finding of Pollard (Science, **103**, 758) and has the following composition: water, 1,000 ml; Difco yeast extract, 3 g; proteose no. 3 peptone, 5 g; lactose, 10 g; agar, 15 g. Adjust to pH 6.9 and add 0.1 ml of tergitol-7 and 2.5 ml of 1 per cent bromthymol blue. Incubate 20 hours at 37 C. *Escherichia* produces yellow colonies surrounded by yellow zones. *Aerobacter* produces greenish yellow "gum drop" colonies, larger than those of *Escherichia* and usually surrounded by yellow zones. Paracoli and other lactose nonfermenters produce colonies usually surrounded by blue zones. A few strains of *Neisseria catarrhalis* grow on the medium but produce minute, rough, blue colonies with blue zones. No other bacteria have been observed. Growths do not "run" like other freshly prepared media. *Proteus* has far less tendency to spread. There is apparently no inhibition of coliforms, thus permitting recovery from minute inocula. The counts are about 30 per cent higher than on other selective media.

New Effective Volatile Antiseptics. *Clara A. Bjernes and S. H. Hutner*, Haskins Laboratories, New York.

Basal Media for Studying the Inorganic Requirements of Purple Bacteria and Green Plants. *S. H. Hutner*, Haskins Laboratories, New York.

Improving the Efficiency of the Phenol Coefficient Test Procedure. *Arthur R. Cade*, Givandan-Delawanna, Inc., Delawanna, New Jersey.

Because the standard phenol coefficient method does not present the facts regarding the presence of viable organisms existing at the end of the test period, investigations were made to determine the fundamental causes for this rather serious fault, and to develop modifications in the test method which might serve to overcome it.

Three reasons were found as basic causes. They were the failure to recognize (1) the germicidal action which goes on in the subculture tubes during incubation; (2) the effect of the "random-sampling error"; and (3) the fact that the loopful transfer fails to pick up (therefore record) certain viable organisms which adhere to the sides of the medication tube.

To overcome these three sources of error, the following recommendations are offered: (1) use the Cade-Halvorson plate count technique in place of broth subculture transfers; (2) increase loop size from the present 4-mm standard to one that delivers approximately 0.03 ml; and (3) apply the author's "swab test" to determine the presence and number of organisms adhering to the sides of the glass.

The Utilization of Thin Paper Discs in the Assay of Amylases. *Marion B. Sherwood*, Wellcome Research Laboratories, Tuckahoe, New York.

The method of using thin filter paper discs as carriers of dilutions of amylase upon starch agar plates was applied to the assay of amylases. One per cent amylases extracted with 20 per cent glycerol in the refrigerator overnight were progressively diluted to 1:125 in 1:5 steps. Three and six-tenths per cent starch in 1 per cent agar of uniform consistency, 20 ml per plate, served as the substrate. A comparison of pancreatin with pancreatic amylase as the standard gave a potency of 40.5 per cent with arrowroot and 39.9 per cent with amioca starch as substrate. An estimate of the inherent precision of the assay was calculated from the analysis of these data. Values approximating 0.1 indicated the high degree of precision. As a further indication of the accuracy of the method, pancreatic amylase partially denatured by urea in aqueous solutions was glycerolated and tested against controls in both this and the Wohlgemuth procedure. Potency values of 49.2 and 24.3 per cent for two urea-treated solutions were obtained by the former method. Corresponding figures by the Wohlgemuth procedure were 51.8 and 25.9 per cent.

An Occurrence of Udder Infections Caused by a Thermoduric Streptococcus. *John S. Bryan*, Walker-Gordon Laborabory Company, Plainsboro, New Jersey.

An increased bacterial count of high quality pasteurized milk was caused by a thermoduric streptococcus infection of the udders of cows. Milk from the infected udders in turn contaminated the equipment, but this contamination or seeding was readily eliminated in routine cleaning and sterilizing after the infected cows were located and removed from the supply. Detection of the infected cows was difficult because the symptoms and physical appearance of the milk were not the same in each case. It was necessary to pasteurize in the laboratory the milk from each cow to confirm our physical checkup findings and to locate the infected cows.

Our experience has not indicated that this thermoduric bacterium normally resides in the udder of the cow.

There is no evidence to indicate that the thermoduric bacteria are important in milk from a public health standpoint, but they are important in milk plant operations from the pasteurized milk bacterial count control angle.

The Practical Significance of So-called Heat-resistant Coliform Organisms in the Coliform Testing of Pasteurized Milk. *Edythe C. Alff and Leon Buchbinder*, Bureau of Laboratories, New York City Department of Health, New York.

An investigation was carried out to determine the role of heat-resistant coliforms in the coliform test of pasteurized milk. A milk plant study of raw, line, and final milk revealed that heat-resistant coliforms are rarely, if ever, found in the final product. A year's survey, comprising 1,000 samples of raw milk, demonstrated that far fewer coliform organisms are usually found in this product than are commonly used in the laboratory pasteurization of coliforms. Determination of the heat resistance of coliforms by one completely submerged technique and two partially submerged techniques revealed that, when reasonable numbers are used, complete kill is uniformly obtained only by the former technique. It is concluded that coliform survivors are not found after laboratory pasteurization of milk when an adequate physical technique and reasonable numbers of organisms are used. Heat-resistant coliforms, therefore, seem to have no practical significance in the coliform testing of pasteurized milk.

STUDIES ON THE SWAB-RINSE TEST FOR FOOD UTENSIL SANITATION. *Leon Buchbinder and Sylvia Mazur*, Bureau of Laboratories, New York City Department of Health, New York.

THE SATURATION OF BACTERIAL LIPIDS AS A FUNCTION OF TEMPERATURE. *Eugene R. L. Gaughran*, Rutgers University, New Brunswick, New Jersey.

A comparative study of the cellular lipids of a mesophilic and a stenothermophilic bacillus was made in the course of an investigation of the elevated minimum temperature for growth of the latter organism.

The data presented indicate that for the mesophile, the total cellular lipid and its constituent fractions decrease in quantity and degree of unsaturation as the temperature of cultivation of the organism is increased, while the lipids of the stenothermophilic bacillus are strikingly constant both in quantity and degree of saturation. The high degree of saturation of the acetone-soluble fats of the stenothermophilic organism over a wide temperature range provides evidence that a relatively large proportion of the total lipids approach solidity as the minimum temperature for growth is reached. The incompatibility of this situation with active metabolism at lower temperatures is quite evident and implies that the consistency of the fats elaborated by stenothermophiles may prevent active metabolism at lower temperatures and fix the minimum temperature for growth.

The nature of the enzyme complement of the thermophilic organism in question appears to be the responsible factor.

INFLUENCE OF PURINES ON THE ANTIBACTERIAL ACTIVITY OF ACRIDINES. *D. Eldridge, E. Titsworth, and C. Unger*, Research Laboratories, Hoffman-LaRoche, Inc., Nutley, New Jersey.

Nucleic acid exerts an antagonistic effect on the antibacterial activity of the acridine dye stuffs, acriflavine and proflavine. This antagonism can be demonstrated by the inhibition of the bacteriostatic activity *in vitro* and the tissue sterilization by topical administration *in vivo*. Adenine and adenylic acid failed to show inhibiting properties.

INFLUENCE OF PURINES ON THE TOXICITY AND ANTITRYPANOSOMAL ACTIVITY OF ACRIDINES. *W. Schleyer, M. Buck, and R. J. Schnitzer*, Research Laboratories, Hoffman-LaRoche, Inc., Nutley, New Jersey.

Nucleic acid detoxified proflavine. It interfered with the trypanocidal activity of acriflavine and proflavine *in vitro*, but did not antagonize the trypanocidal effect *in vivo*.

NONBACILLARY FORMS OF MYCOBACTERIUM TUBERCULOSIS AND MYCOBACTERIUM LEPRAE. *Eleanor Alexander-Jackson*, Cornell University Medical College, New York.

THE INACTIVATION OF INFLUENZA VIRUS BY CERTAIN VAPORS. *Thomas C. Grubb, Marie L. Miesse, and Bruno Puetzer*, Research Laboratories, Vick Chemical Company, Flushing, New York.

IMMUNOLOGICAL STUDIES ON THE MUCOID VARIANTS OF PSEUDOMONAS AERUGINOSA. *Lewis H. Schwarz and Joseph Lazarus*, New York.

This paper deals with the attempt to immunize laboratory animals with the components of the mucoid variant of *Pseudomonas aeruginosa*. Failure of this organism to respond to treatment with streptomycin or other chemotherapeutic agents prompted the experiment. A series of rabbits received, intravenously, graduated amounts of bacterin, heat-treated exotoxin, purified exotoxin, and endotoxin. The animals showed a rapid loss of weight, finally succumbing. Autopsies revealed that all of the animals died of toxemia.

It is therefore concluded that little if any value is to be expected from this method of treatment, and it is our confirmed opinion that streptomycin has little to offer as an antibiotic capable of eradicating all strains of *P. aeruginosa.*

THE PRODUCTION OF ANTIBODIES AGAINST A SYNTHETIC ESTER OF OLEIC ACID. *Gardner Middlebrook and Rene J. Dubos,* Rockefeller Institute for Medical Research, New York.

THE INHIBITION OF EXPERIMENTAL DRUG ALLERGY BY PRIOR FEEDING OF THE SENSITIZING AGENT. *Merrill W. Chase,* Rockefeller Institute for Medical Research, New York.

PENICILLIN AND SULFADIAZINE SENSITIVITY OF 385 STRAINS OF CORYNEBACTERIUM DIPHTHERIAE. *Lillian Buxbaum, Nancy Nenner, and Vera B. Dolgopol,* Willard Parker Hospital, New York.

Three hundred eighty-five strains of *Corynebacterium diphtheriae* were tested *in vitro* for sensitivity to penicillin. Two hundred seventy-eight (72.2 per cent) were sensitive to 0.25 to 1 unit of penicillin. Thus the majority of these organisms are relatively resistant to penicillin as compared with the more sensitive microorganisms such as gonococcus, streptococcus, and staphylococcus.

Sulfathiazole exerted little inhibitory action on the growth of the diphtheria bacillus in concentrations as high as 50 mg per cent.

Penicillin, when administered systematically in doses from 5,000 to 15,000 units, exerted some protective effect on the skin of guinea pigs inoculated intracutaneously with toxin or the diphtheria bacillus.

Penicillin solutions containing 800 to 1,000 units per ml were used to spray the throat and nose of carriers of *C. diphtheriae;* this did not reduce the number of days their cultures remained positive as compared to the controls. The majority of both treated and untreated patients no longer harbored the bacilli after 21 days' hospitalization.

Only those treated patients whose cultures were resistant to 0.5 unit of penicillin before treatment remained positive, but there were several patients in the untreated group who still harbored bacilli sensitive to 0.25 unit. This seems to indicate that penicillin in adequate concentrations will shorten the duration of the carrier state of those individuals who harbor *C. diphtheriae* sensitive *in vitro* to less than 0.5 units of penicillin per ml.

BACTERIOLOGY AND PUBLIC HEALTH. *Israel Weinstein,* Commissioner of Health, New York City.

CHANGES IN THE LABORATORY ASPECTS OF THE VENEREAL DISEASES. *Richard C. Arnold,* Venereal Disease Laboratory, U. S. Public Health Service, Staten Island.

SEROLOGICAL STUDIES OF THE GENUS XANTHOMONAS

I. CROSS-AGGLUTINATION RELATIONSHIPS

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The genus *Xanthomonas* proposed by Dowson (1939) has attracted the attention of many phytopathologists both in this country and abroad. The genus is defined by its author as follows: "*Xanthomonas* n.g., non-sporing, rod-shaped bacteria, gram negative, motile by means of one polar flagellum (rarely two present) or non-motile, yellow in the mass on nutrient agar and on potato, on both of which abundant slimy growths are formed. Most species digest starch and produce acid in lactose. None produces acid in salicin." There seems little doubt that these organisms form a definite group. On the basis of morphology and biochemical properties, however, the individual species have few distinguishing features (Bergey *et al.*, 1939; Dowson, 1939). They are all pathogenic for plants.

Since establishment of the genus, the more than 40 members have assumed a more rational place in bacteriological taxonomy but are still little understood as a group. It is unfortunate that these organisms, which on numerous occasions have been of great economic importance, have received little study. We have scant knowledge of the relationships between the various species and subspecies. Inasmuch as it is the practice of the phytopathologist in general to form a new species if a bacterial plant pathogen of a recognized genus is isolated from a new host, it is apparent that many species could have been duplicated. This practice of basing species differentiation almost solely on host of isolation (with the host range almost always inadequately investigated) has resulted in a seeming increase in the size of the genus, giving rise to the belief among certain workers that all of the members may not represent true species.

As an approach to this problem we have undertaken an extensive serological survey of the group to determine whether the genus could be logically classified in this fashion and to ascertain whether antigenic characteristics could be correlated with host range.

Serological work with species of this genus has been scattered and incomplete. St. John-Brooks, Nain, and Rhodes (1925) found that of three strains of *Xanthomonas campestris* two appeared to act alike, while one was unique in its agglutinative reactions in ten antiserums prepared with bacterial plant pathogens. Two strains of *Xanthomonas malvacearum* showed identical cross reactions. They also stated that a close serological relationship was manifest between the two previously mentioned species (*X. campestris* and *X. malvacearum*) and *X. phaseoli*, *X. phaseoli* v. *sojense*, *X. pelargonii*, and *X. vitians*. On the other hand, *X. pruni*, *X. stewartii*, and *X. hyacinthi* had no relationship with the other species tested.

Sharp (1927) stated that two isolates of *Xanthomonas phaseoli* were alike serologically although differing in some cultural characteristics and virulence. He found that *X. phaseoli* and *X. phaseoli* v. *sojense* could be differentiated serologically.

Link and Link (1928) observed that the agglutination test could be used to differentiate *Xanthomonas malvacearum* from *X. campestris*, *X. phaseoli*, *X. citri*, *X. cucurbitae*, and *X. pruni*. These workers asserted that the direct agglutination test was of little use in distinguishing between *X. malvacearum* and *X. phaseoli* v. *sojense*. They found the former organism to be more closely related to the yellow bean pathogens than to *X. campestris* serologically. It was further concluded by them that not all the yellow organisms tested form a single group serologically. Nevertheless, they did state that *X. campestris*, *X. malvacearum*, *X. phaseoli*, *X. phaseoli* v. *sojense*, and *Corynebacterium flaccumfaciens* apparently constitute a serological group.

In a later publication Link, Edgecombe, and Godkin (1929) utilized agglutinin absorption as well as the agglutination test. It was revealed by these studies that many loose relationships were manifest between the yellow plant pathogens. Of the organisms tested they found *Xanthomonas campestris* and *Bacterium X* (horse radish pathogen = *X. campestris* v. *armoraciae*), *X. phaseoli*, *X. cucurbitae*, *Bacterium Y* (poppy pathogen = *X. papavericola*), *X. pruni*, *X. translucens*, *X. translucens* v. *undulosa*, and *Bacterium Z* (cereal pathogen = *X. translucens* possibly v. *secalis?*) gave varying group reactions. *Bacterium Z*, *X. pruni*, and *X. papavericola* gave the weakest group reactions. *Xanthomonas campestris*, *X. campestris* v. *armoraciae*, *X. cucurbitae*, and *X. papavericola*, they maintained, constituted a group loosely connected antigenically to the other organisms tested. The cereal pathogens made up a closely related group. These workers stated that serological studies apparently gave promise in grouping and classifying at least some of the closely related species, varieties, and subvarieties of phytopathogenic bacteria.

Williams and Glass (1931) and Horgan (1931) independently established the serological homogeneity of *Xanthomonas malvacearum* by agglutinin absorption tests. McNew and Braun (1940) and Braun and McNew (1940) concluded that *X. stewartii* was not serologically homogeneous and that antigenic differences were not correlated with virulence or colony type. Stapp (1938) pointed out that *X. begoniae* was distinguished from *X. campestris* on the basis of the agglutination test. Miller *et al.* (1940) found their *X. corylina* to be serologically distinct from *X. juglandis*, and Hagborg (1942) found comparable agglutination of many *X. translucens* strains in several antiserums of low titer.

MATERIALS AND METHODS

By far the largest contribution to our collection of *Xanthomonas* cultures was made by Dr. Walter H. Burkholder. Dr. Mortimer P. Starr has advanced further specimens. A large number of *X. translucens* isolates were received from both Dr. Jack Wallin and Dr. W. A. F. Hagborg. We are deeply indebted to all of these workers for their co-operation.

Antiserums for the agglutination tests were prepared by intravenous injection in young rabbits. The doses were such that a serum was obtained that had a final homologous agglutinating titer of 1,600 to 6,400. Such antiserums were more easily handled in subsequent absorption experiments than serums of greater titer. The agglutination tests were incubated 18 hours at 45 C. Additional incubation at 4 C did not enhance the titer. Multiple small absorption doses were employed.

ELIMINATION OF MUCOID INTERFERENCE

It is the usual practice of most laboratories working with phytobacteria to utilize media high in carbohydrate. A 1 or 2 per cent sucrose or glucose medium is ordinarily employed. On this sugar-rich medium the organisms usually initiate growth slowly, but in 72 hours growth is abundant at room temperature. The bulk of the growing mass is composed of extracellular mucoid material. There does not seem to be an increase in capsule size. In fact, electron micrographs taken of organisms from the gummy mass failed to demonstrate capsules (unpublished data).

TABLE 1

Unilateral agglutination in Xanthomonas vascularum and Xanthomonas phaseoli groups with heavily mucoid cultures

ORGANISMS AGGLUTINATED	ANTISERUMS PREPARED FOR										
	XV14	XV16	XH1	XI3	XP5	XC4	XV1	XT11	XG3	XP7	XP14
X. vesicatoria XV14	++++[*]	++++	+++	++	+++	++++	+++	+++	+++	++	+++
X. vesicatoria v. *raphani* XV16	+++	+++	+++	+++	+++	++++	+++	+++	+	+++	−
X. hederae XH1	+++	++++	++++	+++	++	+++	++++	+++	++++	++++	+++
X. incanae XI3	++++	++++	++++	+++	++	++++	+++	+++	+++	++	−
X. papavericola XP5	++++	++++	++++	+	++++	++++	+++	+	+++	+++	++
X. campestris v. *armoraciae* XC4	++++	+++	+++	+++	++++	++++	++++	+++	++	+	−
X. vascularum XV1	+++	+++	++++	+++	++	++	+++	++	++	++	++
X. taraxaci XT11	++++	+++	++++	+++	++++	++++	++++	+++	+++	++	++
X. geranii XG3	−	−	−	−	−	−	−	−	++++	++++	++++
X. pelargonii XP7	−	−	−	−	−	−	−	−	++++	+++	++++
X. phaseoli XP14	−	−	−	−	−	−	−	−	+++	+++	++++

[*] In all agglutination tables + = agglutination at 1:50 or 1:100; ++ = agglutination at 1:200 or 1:400; +++ = agglutination at 1:800 or 1:1600; ++++ = agglutination at 1:3200 or greater; − = no agglutination.

During the early phases of the problem organisms were employed that produced an abundance of the gum. It was found, however, that the mucoid material interfered with the antigenic patterns. A large number of unilateral serological relationships were demonstrated in the preliminary work (table 1). This reaction was shown to be in the direction of *Xanthomonas vascularum* group cultures agglutinating in *X. phaseoli* group antiserums. There were other such intergroup reactions also evident throughout the genus. It was found that the

TABLE 2

*Cross-agglutination reactions of 36 species and subspecies of Xanthomonas**

Part A

ORGANISM AGGLUTINATED	ANTISERUMS PREPARED FOR														
	XV14	XV16	XH1	XI3	XP5	XC4	XV1	XT11	XB2	XC10	XG4	XP7	XP14	XM13	XP19
X. vesicatoria XV14	++++†	++++	+++	++	+++	++++	+++	+++	++	+++	−	−	−	−	−
X. vesicatoria v. *raphani* XV16	+++	+++	+++	+++	+++	++++	++++	+++	++	++	−	−	−	−	−
X. hederae XH1	+++	++++	++++	+++	++	+++	++++	+++	++	++	−	−	−	−	−
X. incanae XI3	++++	++++	++++	+++	++	++++	+++	+++	++++	++	−	−	−	−	−
X. papavericola XP5	++++	++++	++++	+	++++	++++	+++	+	++	++	−	−	−	−	−
X. campestris v. *armoraciae* XC4	++++	+++	+++	+++	++++	++++	++++	+++	+++	+++	−	−	−	−	−
X. vascularum XV1	+++	+++	++++	+++	++	++	++++	++	+++	+++	−	−	−	−	−
X. taraxaci XT11	++++	+++	++++	+++	++++	++++	++++	+++	+++	+++	−	−	−	−	−
X. barbareae XB2	++	+++	+	++	+++	++++	+++	++	+++	++++	++++	+++	++	++	+
X. campestris XC10	+++	+++	+++	+++	++	++	+++	++	+++	+++	++++	++++	++	++	++
X. geranii XG4	−	−	−	−	−	−	−	−	++	+++	++++	++++	++++	++	+++
X. pelargonii XP7	−	−	−	−	−	−	−	−	++++	+++	++++	+++	++++	+++	++
X. phaseoli XP14	−	−	−	−	−	−	−	−	+++	++	+++	+++	++++	+++	++
X. malvacearum XM13	−	−	−	−	−	−	−	−	+++	++	+++	+++	++++	++++	+++
X. phaseoli v. *fuscans* XP19	++	++	−	−	−	++	−	−	++	++	++	++	++	+++	++++

* There were no reactions between the organisms listed in table 2, part A and those included in table 2, part B.

† See footnote to table 1 for explanation of symbols.

TABLE 2

Part B

ORGANISM AGGLUTINATED	ANTISERUMS PREPARED FOR																				
	XB3	XC6	XJ1	XT1	XT8	XT9	3055	2049	3045	XC11	XM16	XP13	XC12	XP21	XL2	XH3	XV18	XM12	XG9	XH7	XR1
X. begoniae XB3	++++	+++	+	+++	++	+++	+++	+++	++	++	+++	–	–	–	–	–	–	–	–	–	–
X. cucurbitae XC6	+++	++++	+	+++	++	+++	+++	+++	+++	+	+++	–	–	–	–	–	–	–	–	–	–
X. juglandis XJ1	+	++	+++	++	+++	++	+	+	+	++	+	–	–	–	–	–	–	–	–	–	–
X. translucens XT1	+++	++++	+++	++++	+++	+++	++++	+++	++	+++	+++	–	–	–	–	–	–	–	–	–	–
X. translucens f.sp. *cerealis* Race 2 XT8	+++	++++	+	++++	+++	+++	+++	+++	+++	++	+++	++	–	–	–	–	–	–	–	–	–
X. translucens f.sp. *cerealis* Race 1 XT9	++	+++	+	+++	+++	+++	+++	+++	+++	+	+++	+	–	–	–	–	–	–	–	–	–
X. translucens f.sp. *hordei-avenae* 3055	+++	+++	+	++++	+++	+++	+++	+++	+++	+	++	–	–	–	–	–	–	–	–	–	–
X. translucens f.sp. *hordei* 2049	++	++	+	+++	+++	+++	++++	++++	+++	++	+++	++	–	–	–	–	–	–	–	–	–
X. translucens f.sp. *undulosa* 3045	++	+++	+	++	+++	+++	+++	++++	+++	++	++	+	–	–	–	–	–	–	–	–	–
X. carotae XC11	+	++	+++	++	++	+	+++	++	+	+++	++	–	–	–	–	–	–	–	–	–	–
X. maculafoliumgardeniae XM16	++	+++	+	+++	++	+++	+++	+++	++	+	+++	+	–	–	–	–	–	–	–	–	–
X. pruni XP13	–	–	–	–	–	–	–	–	–	–	–	++++	++++	+++	+++	–	–	–	–	–	–
X. corylina XC12	–	–	–	–	–	–	–	–	–	–	–	++++	++++	++++	+++	–	–	–	–	–	–
X. phaseoli v. *sojense* XP21	–	–	–	–	–	–	–	–	–	–	–	++++	+++	+++	+++	–	–	–	–	–	–
X. lespedezae XL2	–	–	–	–	–	–	–	–	–	–	–	++++	+++	++++	+++	–	–	–	–	–	–
X. holcicola XH3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	–	–	–	–	–
X. vignicola XV18	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++++	–	–	–	–
X. manihotis XM12	–	–	–	–	–	–	–	–	–	–	–	++	–	–	–	–	–	+++	–	–	–
X. gummisudans XG9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	–	–
X. hyacinthi XH7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++++	–
X. rubrilineans XR1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++++

gummy material, extracted with warm saline and harvested according to the method of Morgan and Beckwith (1939), yielded a large quantity of polysaccharide. In precipitin tests this carbohydrate acted as a common component giving strong reactions at high serum concentrations. By the use of extracted polysaccharide substances the previously mentioned unilateral relationships could be made bilateral, with high concentrations of both antiserum and polysaccharide. The cross reactions with *Xanthomonas*-derived polysaccharides were essentially the same as those obtained by Morgan and Beckwith with *Salmonella*. We have not investigated the immunochemical properties of the gum inasmuch as this phase of the *Xanthomonas* problem is being studied in the laboratory of Dr. M. P. Starr (personal communication).

If, after extraction with warm saline, the cells were several times washed and resuspended in saline and then used for the agglutinating antigen, the unilateral relationship was not evident. This loss was of the wide group relationships caused by the extracellular mucoid material.

Organisms growing in medium lower in sugar content produced meager growth unless the nutrients to supply the nitrogenous needs were made readily available. We have employed a brain-heart infusion agar (Difco) which contains 0.2 per cent glucose. By inoculating agar slopes heavily and incubating 18 hours at 28 C we obtained abundant growth. Such cultures apparently approach a true "S" phase. Continued growth on this medium produces an increasing amount of mucoid substance. Suspensions made from 18-hour cultures and employed in agglutination tests obliterated the one-sided reactions, as had the extracted cells. It was with these smooth, polysaccharide-reduced cultures that the results herein reported were obtained.

EXPERIMENTAL RESULTS

The results of cross-agglutination reactions of 36 *Xanthomonas* species and subspecies and their corresponding antiserums are best presented in a composite table (table 2). It is obvious that several definite serological blocks are formed, composed of 2 to 11 cultures. These blocks are referred to as serological groups. Elimination of most of the mucoid factor has left little in the way of intergroup response. Absorption experiments, to be described later, demonstrate that common components exist in a group, and that usually subgroups may be determined on specific characters.

Two organisms, *Xanthomonas campestris* (XC10) and *X. barbareae* (XB2), hold a unique position in that they are antigenically closely related to two compact serological groups. One of the largest of these is the "*X. vascularum* group" which is composed of *X. vascularum*, *X. vesicatoria*, *X. vesicatoria* v. *raphani*, *X. hederae*, *X. incanae*, *X. papavericola*, *X. campestris* v. *armoraciae*, and *X. taraxaci*. The *X. campestris* group (XC10 and XB2) reacts reciprocally with all of the species listed above. A smaller group, similarly related to the *campestris* pair but unrelated to the *vascularum* group, is comprised of *X. phaseoli*, *X. phaseoli* v. *fuscans*, *X. geranii*, *X. pelargonii*, and *X. malvacearum*. This *phaseoli* block shows strong antigenic ties to one another and to *X. campestris* and *X.*

barbareae. The "*fuscans*" variety agglutinates in many "*vascularum*" antiserums, but the reciprocal is not true.

The largest serological block is formed by members of the *Xanthomonas translucens* group. All available species and subspecies of *X. translucens*, *X. maculafoliumgardeniae*, *X. cucurbitae*, *X. carotae*, *X. juglandis*, and *X. begoniae* belong in this group. *X. juglandis* and *X. carotae* show definite, but less strong, affinities for the other members. Subsequent absorption experiments indicate that many of these so-called species and subspecies are serologically identical, independent of source of isolation (Elrod and Braun, 1947).

The *Xanthomonas pruni* group is composed of *X. pruni*, *X. corylina*, *X. phaseoli* v. *sojense*, and *X. lespedezae*. These interactions are very strong, heterologous reactions often equaling the homologous titer. This block is joined to the *translucens* group through minor reactions with *X. pruni* antiserum by certain *X. translucens* cultures and *X. maculafoliumgardeniae*.

By means of the agglutination tests with mucoid-depleted cultures it has not been possible to place 6 of the species or subspecies in a group. Of these, 3 were doubtful xanthomonads. Two isolates of *Xanthomonas hyacinthi* and 2 of *X. rubrilineans* did not exhibit certain characteristics usually associated with the genus. They failed to lypolyze fat (Starr and Burkholder, 1942), formed ammonia from urea (unpublished data), and did not appear in mass as do typical cultures. *X. manihotis*, represented by 6 isolates, was gray white in mass rather than yellow. The latter organism did react with *X. pruni* antiserum, but the reciprocal reaction did not occur. *X. gummisudans* was apparently typical but showed no serological affinity with any of the other organisms under study.

The two remaining organisms, *Xanthomonas vignicola* and *X. holcicola*, have proved an enigma. If allowed to form an abundance of gum, they show strong serological affinity to one another and also to some members of the *vascularum* group. However, cells devoid of most of the polysaccharide were almost specific. They have not been placed in any group.

DISCUSSION

So-called biochemical methods employed in an effort to differentiate the species in the genus *Xanthomonas* are admittedly inadequate. This becomes readily apparent when one attempts to identify an organism from an unknown host by these physiological characteristics. The specific traits described are more often generic in scope (Starr, 1946; Dowson, 1939; Starr and Burkholder, 1942). The result has been the establishment of species based solely on source of isolation, the latter often considered the only host. Unfortunately, the extensiveness of the host range was seldom examined, or if investigated, only within narrowly specified botanical limits. Little consideration was given to the possibility of a particular species' being pathogenic over a range of taxonomically distinct hosts. A taxonomic system based on such false premises is not only misleading but almost useless. We do not propose, however, to establish a taxonomy in the genus based solely on serological characteristics. It is believed that due cognizance should be taken of inheritable variations of pathological

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SEROLOGICAL STUDIES OF THE GENUS XANTHOMONAS

II. XANTHOMONAS TRANSLUCENS GROUP

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It was demonstrated in a previous paper (Elrod and Braun, 1947) that the genus *Xanthomonas* could be divided into five immunologically distinct groups plus several apparently unrelated species. Of the six organisms which did not fall into any group, three were doubtful *Xanthomonas* cultures. The group with the greatest membership was called the *Xanthomonas translucens* group. It is the purpose of this paper to report on the serological relationship of each member of the latter group, to demonstrate the immunological homogeneity of certain members, and to elucidate specific entities within this large group.

ORGANISMS COMPRISING THE XANTHOMONAS TRANSLUCENS GROUP

The species and subspecies that make up this group have a wide and varied host range. In table 1 is presented information concerning natural hosts, the isolates available for study, and the original authority for the species in question. The host range of the members comprising the group is wide, covering many botanically distinct families. The *Xanthomonas translucens* cultures proper have a reported host range within the cultivated grasses. An attempt has been made to present information upon which some workers have split the species into *forma specialis* and even races of a *forma specialis*. Other members do not, according to the literature, infect the grasses. *Xanthomonas juglandis* is considered specific on walnut, *X. carotae* on carrot, *X. begoniae* on begonia, *X. cucurbitae* on squash and field pumpkin, and *X. maculafoliumgardeniae* on the gardenia.

EXPERIMENTAL RESULTS

In table 2 are presented data on the cross-agglutination tests conducted with 11 members of the serological group under discussion (Elrod and Braun, 1947). Arbitrarily selected on previous knowledge concerning host, these strains represent (except for some *forma specialis* and races of *Xanthomonas translucens*) the majority of the recognized species and subspecies of the group. In addition to the members of the species *X. translucens* there are many species isolated from plants far removed from the grasses. Nevertheless, isolates XM16 (*X. maculafoliumgardeniae*), XB3 (*X. begoniae*), XC6 (*X. cucurbitae*), XJ1 (*X. juglandis*), and XC11 (*X. carotae*) all show varying degrees of immunological reactivity within themselves and the *X. translucens* organisms. All reactions were marked, albeit some stronger than others.

TABLE 1

Species and subspecies with representative hosts of the Xanthomonas translucens group

SPECIES OR SUBSPECIES	ISOLATES STUDIED	NATURAL HOST OR HOSTS	AUTHORITY
X. juglandis	XJ1, XJ3, XJ4, XJ5	Walnut (*Juglans* spp.)	Pierce (1901)
X. carotae	XC11, XC41, XC42	Carrot (*Daucus carotae* v. *sativa*)	Kendrick (1934)
X. begoniae	XB3, XB7, XB8, XB11	Begonia (*Begonia* spp.)	Takimoto (1934)
X. cucurbitae	XC6	Squash (*Cucurbita* spp.)	Bryan (1926)
X. maculafoliumgardeniae	XM16	Gardenia (*Gardenia*)	Ark and Barrett (1946)
X. translucens f. sp. *hordei*	2049, XT1	Barley (*Hordeum* spp.)*	Jones *et al.* (1917) Hagborg (1942)
X. translucens f. sp. *undulosa*	3045, XT17, XT4	Wheat, rye (*Triticum* spp., *Secale cereale*)†	Smith *et al.* (1919)
X. translucens f. sp. *hordei-avenae*	3055, XT7, XT16	Barley (*Hordeum* spp.)‡	Hagborg (1942)*†‡§
X. translucens f. sp. *cerealis*	3061	Wheat (*Triticum*)§	Hagborg (1942)
Race 1	XT9	‖	Wallin (1946)‖
Race 2	XT8		Wallin (1946)‖
Race 4	20-1, XT13		Wallin (1946)‖
Race 5	10b, XT12		Wallin (1946)‖
Race 6	60-1, XT15		Wallin (1946)‖

* Infects by wound inoculation *Hordeum* spp., but not *Triticum* spp., *Avena* spp., or *Secale cereale*.

† Infects by wound inoculation *Triticum* spp., *Hordeum* spp., and *Secale cereale*, but not *Avena* spp.

‡ Infects by wound inoculation *Hordeum* spp. and *Avena* spp., but not *Triticum* spp. or *Secale cereale*.

§ Infects by wound inoculation *Triticum* spp , *Hordeum* spp., *Avena* spp., and *Secale cereale*.

‖ Race 1 is pathogenic on Marion and Erban oats, does not infect *Bromus popovii* nor *B. pumpellianus*; race 2 infects Marion and Erban oats, but does not infect *B. inermis* "var. Fischer," *B. popovii*, *B. pumpellianus*, *B. tomentellus*, and *B. tectorum*; race 4 is pathogenic on Boone, Marion, Clinton, and Erban oats and all *Bromus* spp.; race 5 is pathogenic on Boone, Marion, Tama, Clinton, C.I. 4301, Erban, and 4327, but does not infect *B. inermis* "var. 951" and "var. Fischer," nor *B. popovii*; race 6 is pathogenic on Marion, Clinton, C.I. 4301, Erban, and C.I. 4327 and all thirteen *Bromus* spp.

TABLE 2

Cross-agglutination reactions in Xanthomonas translucens group

ORGANISM AGGLUTINATED	ANTISERUMS FOR										
	XB3	XC6	XJ1	XT1	XT8	XT9	3055	2049	3045	XC11	XM16
X. begoniae (XB3)	++++	+++	+	+++	++	+++	+++	+++	++	++	+++
X. curcurbitae (XC6)	+++	++++	+	+++	++	+++	+++	+++	+++	+	+++
X. juglandis (XJ1)	+	++	+++	++	+++	++	+	+	+	++	+
X. translucens (XT1)	+++	++++	+++	++++	+++	+++	++++	+++	++	+++	+++
X. translucens v. *cerealis* race 2 (XT8)	+++	++++	+	++++	+++	+++	+++	+++	+++	++	+++
X. translucens v. *cerealis* race 1 (XT9)	++	+++	+	+++	+++	+++	+++	+++	+++	++	+++
X. translucens v. *hordei-avenae* (3055)	+++	+++	+	++++	+++	+++	++++	+++	+++	+	++
X. translucens v. *hordei* (2049)	++	++	+	+++	+++	+++	++++	++++	+++	++	+++
X. translucens v. *undulosa* (3045)	++	+++	+	++	+++	+++	+++	++++	+++	++	++
X. carotae (XC11)	+	++	+++	++	++	+	+++	++	+	+++	++
X. maculafoliumgardeniae (XM16)	++	+++	+	+++	++	+++	+++	+++	++	+	+++

+ = Agglutination at 1:50 or 1:100.
++ = Agglutination at 1:200 or 1:400.
+++ = Agglutination at 1:800 or 1:1,600.
++++ = Agglutination at 1:3,200 or above.

In order to obtain a truer understanding of this group relationship absorption experiments were performed. Random sampling of absorbing combinations early suggested that the bulk of the isolates could be placed in two large groups, while two other distinct immunological entities were demonstrable.

Absorption of XC6 antiserum by XB3 or XT1 completely obliterated the homologous reaction and, of course, all heterologous reactions (table 3). Mirror absorption with all three of these cultures proved them to be serologically identical. Absorption of XC6 serum with XT8 or 2049 removed all heterologous agglutinins but left antibodies distinct for XC6, XB3, and XT1. By the use of XJ1 and XC11 for absorption, only the reagent responsible for agglutinating these two organisms was removed.

TABLE 3

Absorption experiments in the Xanthomonas translucens group

SERUM	ABSORBED WITH	AGGLUTINATION TITER WITH CULTURE									
		XB3	XC6	XJ1	XT1	XT8	XT9	3055	2049	XC11	XM16
XC6	XB3	−	−	−	−	−	−	−	−	−	−
	XJ1	+++	++++	−	++++	++++	++++	+++	+++	−	++
	XT8	++	+++	−	++	−	−	+++	−	−	−
	XC11	++	++	−	++	++	++	++	++	−	++
	XT1	−	−	−	−	−	−	−	−	−	−
	2049	+++	+++	−	++	−	−	+++	−	−	−
XT8	XC6	−	−	−	−	+++	+++	−	++	−	+
	XJ1	++	+++	−	+++	+++	+++	++	+++	−	+++
	XT9	−	−	−	−	−	−	−	−	−	−
	XC11	+	+	−	++	+++	+++	++	+++	−	++
	XT1	−	−	−	−	+++	++	−	+++	−	+++
	XM16	−	−	−	−	−	−	−	−	−	−
XC11	XT1	−	−	++	−	−	−	−	−	+++	−
	XT8	−	−	++	−	−	−	−	−	+++	−
	XJ1	+	+	−	++	++	++	++	++	+++	++
	XT8 and XJ1	−	−	−	−	−	−	−	−	++	−
XJ1	XT1	−	−	+++	−	−	−	−	−	+++	−
	XT8	−	−	++++	−	−	−	−	−	+++	−
	XC11	−	−	++	−	−	−	−	−	−	−

− = No agglutination at 1:50.
+ = Agglutination at 1:50 or 1:100.
++ = Agglutination at 1:200 or 1:400.
+++ = Agglutination at 1:800 or 1:1,600.
++++ = Agglutination at 1:3,200 or above.

Similar experiments employing XT8 antiserum (table 3) produced comparable results. Absorbing this serum with XC6 left a specific factor for XT8 shared by other members—XT9, 2049, and XM16. Reciprocal absorptions showed the latter organisms to be serologically identical. XJ1 and XC11 had the same effect on XT8 antiserum as they had on XC6 serum and removed only XJ1 and XC11 group factors. With *Xanthomonas carotae* (XC11) antiserum, organisms of the XC6 subgroup and XT8 subgroup removed all agglutinins except those that reacted with XC11 and XJ1. Absorbing with XJ1 left XC11 serum with antibodies capable of reacting against all but XJ1. However, by using both XJ1 and any of the XC6 or XT8 subgroup organisms, a serum was obtained specific for *X. carotae* (table 3).

An antiserum reacting specifically against *Xanthomonas juglandis* was obtained by absorbing XJ1 serum with XC11. Absorption by XC6 or XT8 or equivalent left considerable antibody that reacted with XC11 as well as XJ1 (table 3).

It was observed in the cross-agglutination studies of the entire genus that certain *Xanthomonas translucens* group organisms had the ability to agglutinate to a slight degree in *X. pruni* antiserum. It was revealed by these absorption experiments that only those members of the XT8 subgroup have the capacity of reacting in *X. pruni* antiserum (table 4).

TABLE 4

Serological grouping of Xanthomonas translucens and related cultures in specifically absorbed serums and Xanthomonas pruni antiserum

TESTED WITH	NO. OF CULTURES	SERUM				
		XC6	XT8	XJ1	XC11	*X. pruni*
		Absorbed with				
		XT8	XC6	XC11	XJ1 and XT8	
X. juglandis	4	−	−	+	−	−
X. carotae	3	−	−	−	+	−
X. begoniae	4	+	−	−	−	−
X. cucurbitae	1	+	−	−	−	−
X. maculafoliumgardeniae	1	−	+	−	−	+
X. translucens f. sp. *hordei*	1	+	−	−	−	−
	1	−	+	−	−	+
X. translucens f. sp. *undulosa*	3	−	+	−	−	+
X. translucens f. sp. *hordei-avenae*	3	+	−	−	−	−
X. translucens f. sp. *cerealis*	9	−	+	−	−	+

+ = Agglutination.
− = No agglutination.

By utilizing the four specifically absorbed antiserums, XC6 absorbed with XT8, XT8 absorbed with XC6, XC11 absorbed with XJ1 and XT8, and XJ1 absorbed with XC11; and testing all available isolates (30) of the group, it was possible to locate all specifically. The four *Xanthomonas juglandis* isolates agglutinated in only the specifically absorbed XJ1 antiserum. The three *X. carotae* cultures agglutinated only in the specific XC11 immune serum. Four isolates of *X. begoniae*, one of *X. cucurbitae*, one *X. translucens* f. sp. *hordei*, and three *X. translucens* f. sp. *hordei-avenae* were acted upon only by the specific XC6 serum. All other cultures reacted only in the XT8 specifically absorbed serum. These were one strain of *X. maculafoliumgardeniae*, one *X. translucens* f. sp. *hordei*, three cultures of *X. translucens* f. sp. *undulosa*, and all nine isolates of *X. transucens* f. sp. *cerealis*, including the races of Wallin.

DISCUSSION

Without the aid of the corresponding pathogenicity tests it is difficult to draw definite conclusions. Nevertheless, the implications gained are that in the group of xanthomonads under study many are serologically alike. It would be the tendency of the bacteriologist-serologist to classify serologically identical organisms in the same species.

Inasmuch as the host ranges of the strains found to be identical have never been completely determined, it cannot be said with certainty that they are host-specific. The original description of the alleged species and subspecies in the genus *Xanthomonas* has in almost every instance failed to consider the possibility that the same *Xanthomonas* species can infect a wide variety of taxonomically distinct hosts.

This practice of forming a species solely on the basis of host of original isolation is bacteriologically unsound. Such a method would fail to take into account avirulent or attenuated strains. Species rank cannot be based purely on the foundation that no *Xanthomonas* cultures have previously been described from a similar pathological syndrome on a given host. The value of these data in solving the problem of bacterial identity is open to question.

The results indicate that *Xanthomonas juglandis* is serologically a valid species. Miller *et al.* (1940) found, too, that they were able to distinguish this organism from *X. corylina* and *X. phaseoli* on the basis of straight agglutination tests. Also, *X. carotae* is serologically distinguishable from other members of the group. In this regard it should be emphasized that only those organisms described as *X. juglandis* and *X. carotac* based on source of isolation react in these specific serums.

The remaining organisms in the group are capable of being divided into two serological groups. The *Xanthomonas cucurbitae* group contains the single isolate of *X. cucurbitae*, four isolates of *X. begoniae*, one strain of *X. translucens* f. sp. *hordei*, and three of *X. translucens* f. sp. *hordei-avenae*. Not only do these organisms fall into this specific group, but all are serologically identical as determined by absorption. Experiments are being conducted at present to determine whether all members have the same experimental host range. If such is the case, the many members may logically be reduced to a single species without the need of subspecies designations.

The remaining organisms make up the "*cerealis*" group. All recognized strains of this entity are serologically identical. In this group are found one strain of *Xanthomonas maculafoliumgardeniae*, one of *X. translucens* f. sp. *hordei*, three isolates of *X. translucens* f. sp. *undulosa*, and nine strains of *X. translucens* f. sp. *cerealis*.

It is obvious that the agglutination test suggested by Hagborg (1946) as a method for the rapid identification of *Xanthomonas translucens* cultures must be reconsidered. As is true of any serological method of analysis, the over-all problem must be understood before specific claims can be made.

It is possible, therefore, to limit serologically the *Xanthomonas translucens* group to four distinct immunological entities. Two of these, *X. carotae* and *X. juglandis*, are not only distinct serologically but apparently are host-specific. In light of this, it is conceivable that the two other subgroups may also be restricted in their host range. This is not to say that the antigenically distinct entities will not be added to, or that the problem is as simple as has been suggested. One long-recognized variety, *X. translucens* v. *secale*, and a newly formed one, *X. translucens* v. *phlei-pratense*, were not available for this study.

SUMMARY

By means of absorbed antiserums it is possible to place the available members of the *Xanthomonas translucens* group in four serological subgroups.

Xanthomonas juglandis and *X. carotae* have been shown to be immunologically distinct. A subgroup is indicated for each of these organisms.

All of the other cultures under study fell into two additional subgroups. One, the "*cucurbitae*" group, was comprised of *Xanthomonas cucurbitae*, *X. begoniae*, and some *X. translucens* cultures. *Xanthomonas maculafoliumgardeniae* and the majority of the *X. translucens* strains composed the "*cerealis*" group. The latter organisms all agglutinate in unabsorbed *X. pruni* antiserum.

Reciprocal absorption tests between organisms within each of the subgroups have shown, in each instance, that the isolates formulating a subgroup are alike.

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A STUDY, WITH THE HIGH-VOLTAGE ELECTRON MICROSCOPE, OF THE ENDOSPORE AND LIFE CYCLE OF BACILLUS MYCOIDES

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In spite of numerous investigations during the last seventy years, the structure of the endospore remains one of the dark phases of bacterial cytology. There is no agreement, for instance, about the number of coats which surround the endospore, the existence of a germ pore, the origin of the wall of the germ cell, or the make-up of the spore protoplasm. There is even less agreement on the processes involved in the formation and differentiation of the endospore.

To the authors' knowledge, there has been no systematic study of the endospore with the electron microscope. Such studies have probably been discouraged by the high density of the spore; this has also been the chief obstacle in the way of studying its structure with the light microscope.

ORGANISM AND METHODS

In undertaking the present study, we realized that the power of penetration is a critical factor; we, therefore, used the high-voltage, RCA electron microscope, usually at an accelerating potential of 150 kv. A few observations were made with the commercial model known as the EMU (50 kv), mostly for comparative purposes.

The organism used is strain C_2 of *Bacillus mycoides*. Endospores of this organism were harvested from cultures on glucose agar slants and were washed several times with sterile, distilled water. A drop of the final suspension was placed on a hard layer of nutrient agar in a petri plate, near the periphery, and allowed to run on the surface of the agar by tilting the plate. The boundary of the course followed by the drop was marked, with a wax pencil, on the bottom of the petri plate. A number of such cultures were prepared and incubated at 35 C. Periodically, a plate was taken out of the incubator, a clean cover glass dropped on one of the inoculated areas, slightly pressed down, and carefully lifted with forceps. The under surface of the cover glass carried many of the bacterial elements but very little of the material of the medium. The cover glass was then inverted so that the surface which was in contact with the agar culture became the top surface. A drop of distilled water was placed on that surface and allowed to stand for about a minute with or without a slight periodic tilting to promote suspension of the bacterial elements. During the first two or three hours, a very small loopful of this drop placed on a collodion-covered screen gives a preparation unusually clear and suitable for observation with the microscope. In later hours, the drop suspension may be too concentrated and may be diluted by mixing a loopful thereof with another drop of distilled water on another cover glass. This technique proved to be superior to several other ones tested. It reduces manipulation of the cells and disturbance of the medium to a minimum, and avoids possible loss of important elements and relationships which often results from centrifuging.

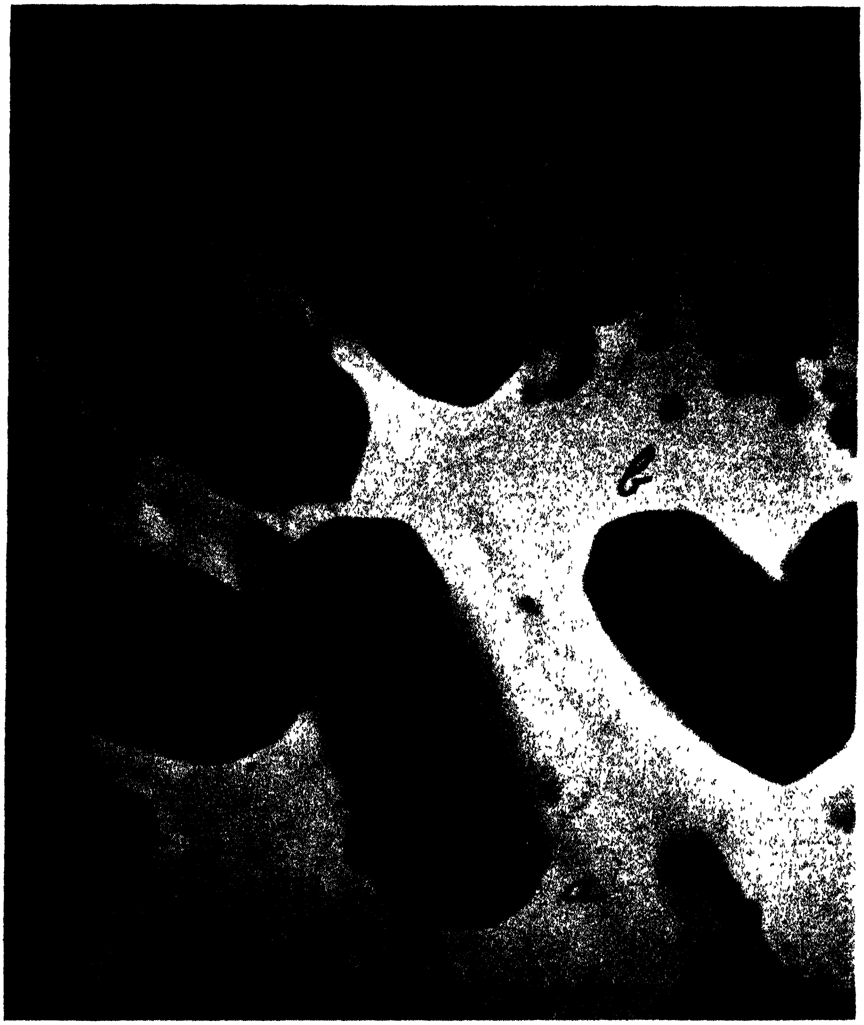


FIG. 1. Endospores from a culture, 10 days old at room temperature, on a slant of nutrient agar. Spore *a* shows transparent, lateral regions and folding of the coats, and spore *b* a slightly shrunken protoplasm; the other three are homogeneously opaque. Photographed at 150 kv. Length of scale = 1 μ.

OBSERVATIONS

Appearance of the endospores. It is usually taken for granted that endospores are shiny bodies of high resistance to deleterious agents. Only in recent years was it pointed out (Hodge and Knaysi, 1937; Knaysi, 1945*a*) that, in the same mother culture, there are endospores of high and low refringence and of high and low resistance to heat. The present study shows that endospores obtained from the same slant culture may be widely different in appearance. On this basis, they

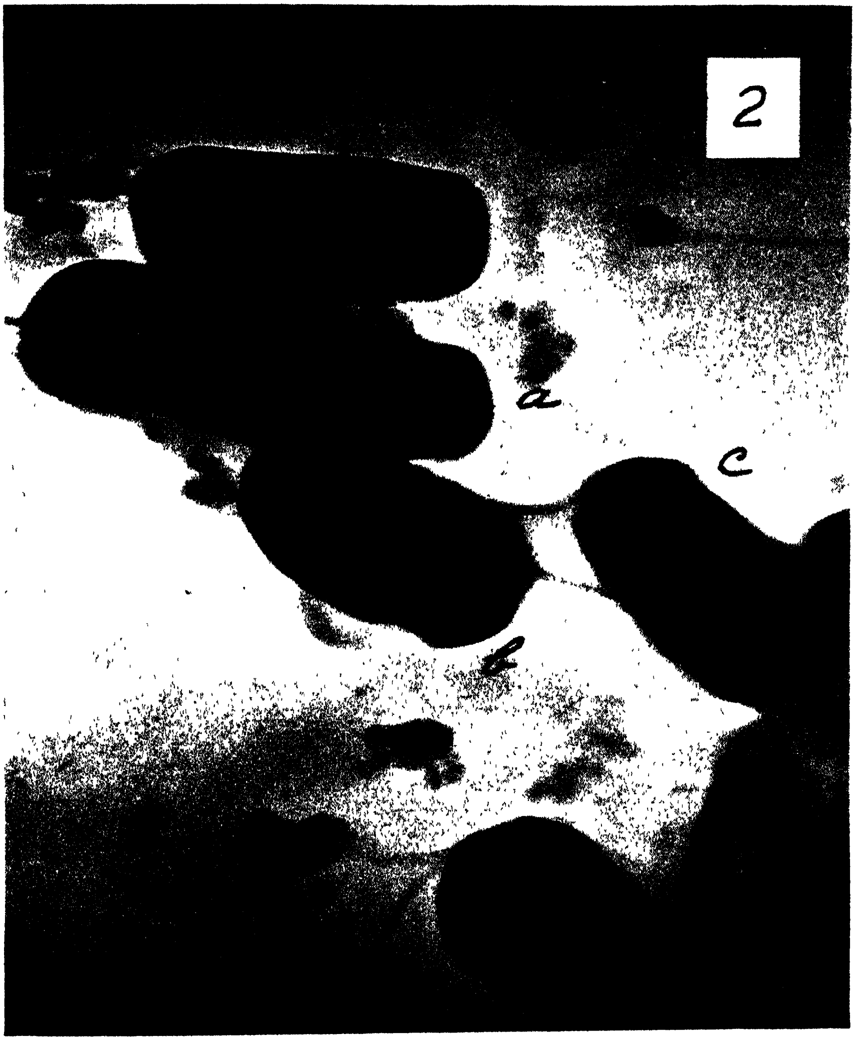


FIG. 2. Material as in figure 1. All spores show a slightly shrunken protoplasm. Spore *a* clearly shows the two coats. Note high elasticity of the outer coat of spores *b* and *c*. Length of scale = 1 μ.

may be classified in three groups: (*a*) those which are homogeneously opaque to the electrons and have a smooth or slightly rough surface (figure 1); (*b*) those which consist of a homogeneously opaque, shrunken protoplasm surrounded by a thick, opaque coat (the shrinkage of the protoplasm is greatest in length, and the thickness of the coat is greatest at the poles; the state of the surface is similar to that of the first group) (figure 2); and (*c*) those which show transparent, lateral areas. Sometimes the entire lateral region is transparent; such spores often show opaque lateral folds and apparent collapse at the transparent regions (figures 1*a*, 3, and 8).

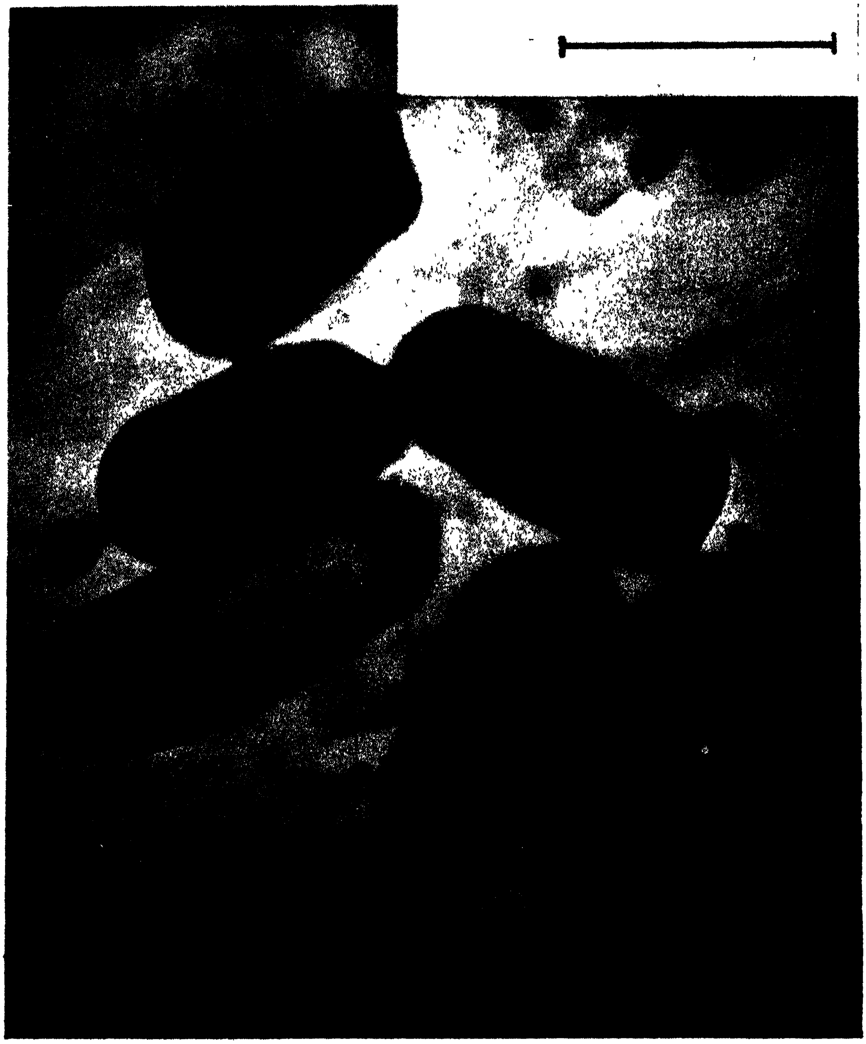
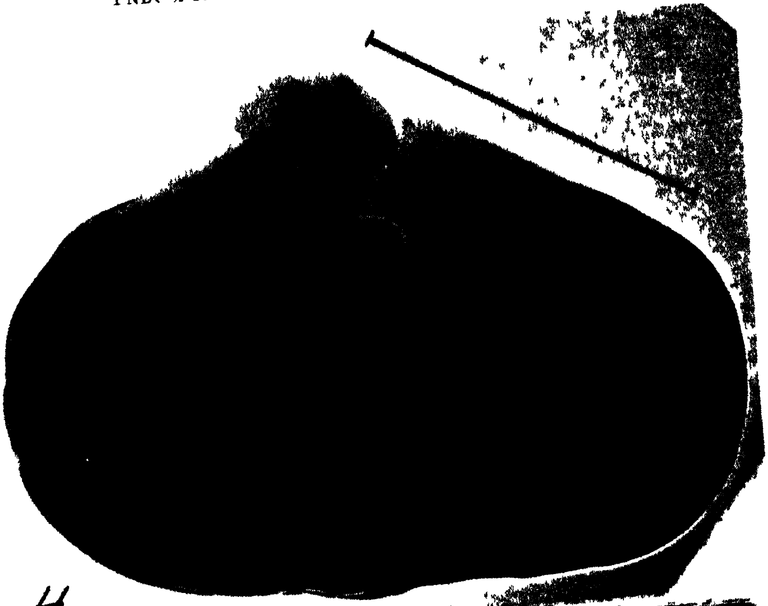


FIG. 3. Material as in figure 1. Spores *a* and *b* show transparent, central regions. Length of scale = 1 μ.



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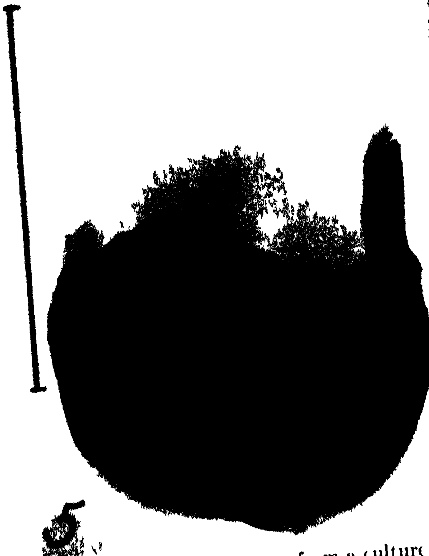

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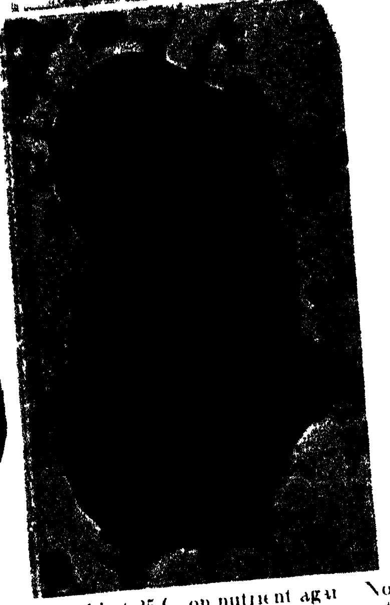
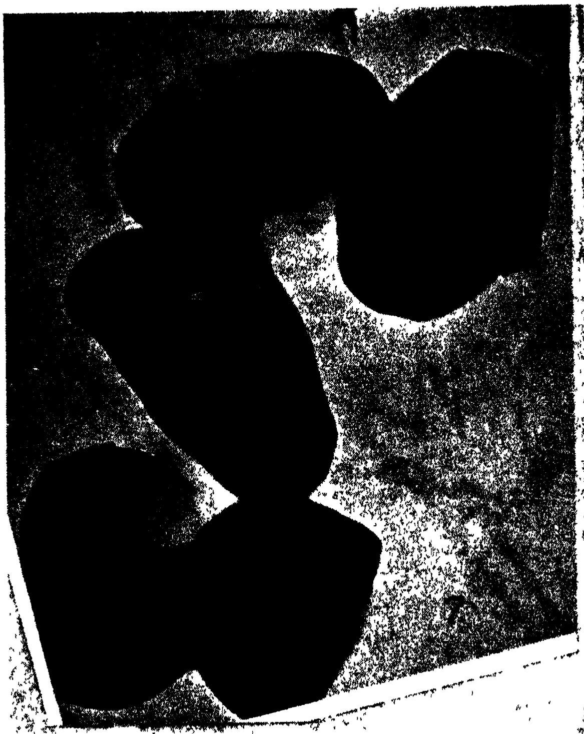


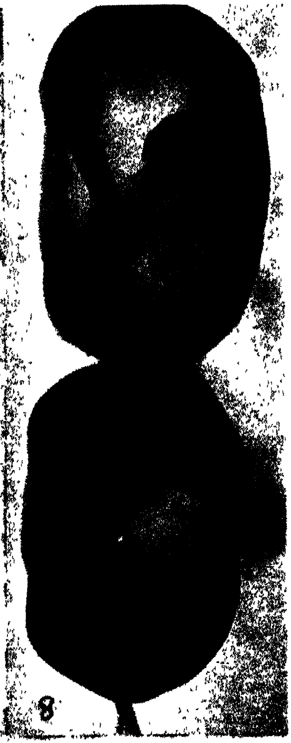
FIG. 4. A germinating spore from a culture 1 hour old at 35 C. on nutrient agar. Note that the inner coat is broken and the outer one has just been torn. Note also the greater elasticity of the outer coat.

FIG. 5. A germinating spore from a culture 2 hours old at 35 C. on nutrient agar. Note the two coats to the right. Photographed at 150 kv.

FIG. 6. A germinating spore from a culture 1½ hours old at 35 C. on nutrient agar. Note the wall of the germ cell. Photographed at 50 kv.

Length of scales = 1 μ.





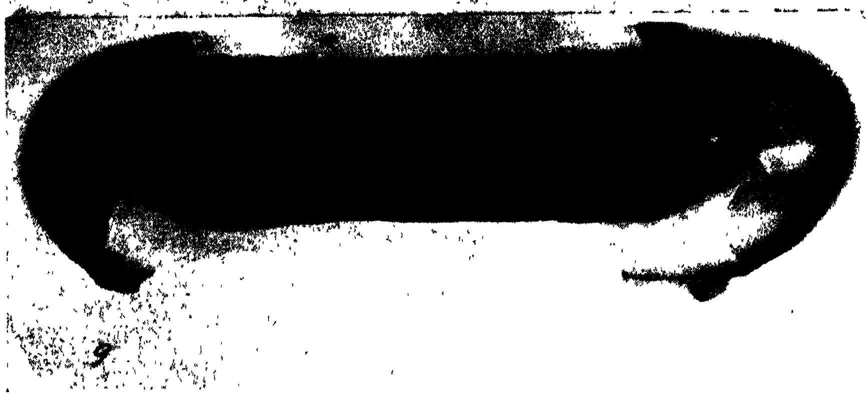


FIG. 7. Germinating spore from a culture, 1 hour old at 35 C, on nutrient agar. Note the unilateral bulging of all spores except *a*. Spore *a* shows swelling all around. Photographed at 150 kv.

FIG. 8. Spores from a culture, 1 hour old at 35 C, on nutrient agar. Note the transparent regions in both spores and the folded coat in the upper one. Photographed at 150 kv.

FIG 9. A germ cell carrying the two halves of the completely severed coats as caps around its ends. From a culture, 1$\frac{3}{4}$ hour old at 35 C, on nutrient agar. Photographed at 150 kv.

Length of scales = 1 μ.

Structure of the endospore. The spore protoplasm appears homogeneous. The reason for this, according to Knaysi and Baker (1947), is the presence of ribo-

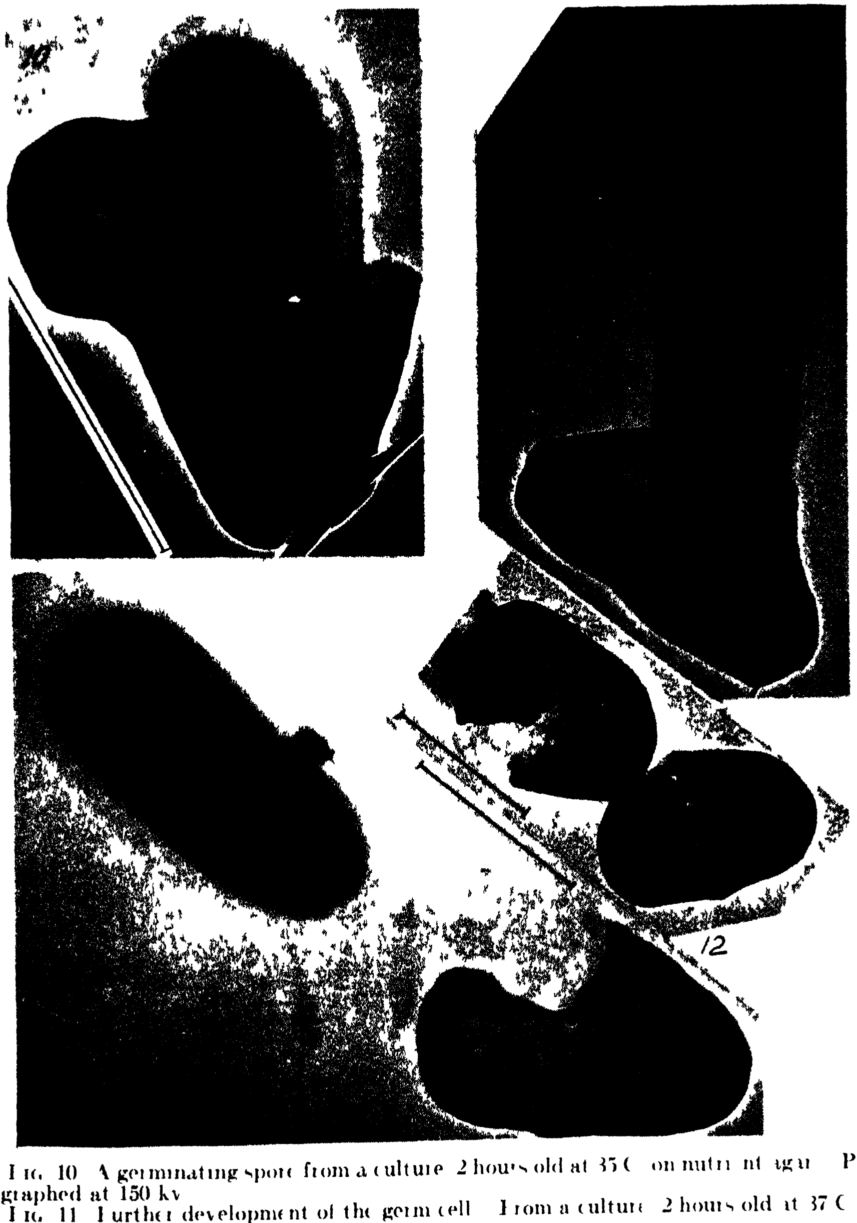


FIG. 10. A germinating spore from a culture 2 hours old at 35 C. on nutrient agar. Photographed at 150 kv.

FIG. 11. Further development of the germ cell. From a culture 2 hours old at 37 C. on nutrient agar. Photographed at 150 kv.

FIG. 12. Two empty spore coats. Photographed at 150 kv.

FIG. 13. A vegetative cell and an empty spore coat. The stubbiness of the cell indicates that it is a freshly liberated germ cell. Photographed at 150 kv.

Length of scales = 1 μ.

nucleic acid diffused throughout the cytoplasm. The protoplasm is surrounded by two coats. The inner one of these coats is the more rigid, and the outer one

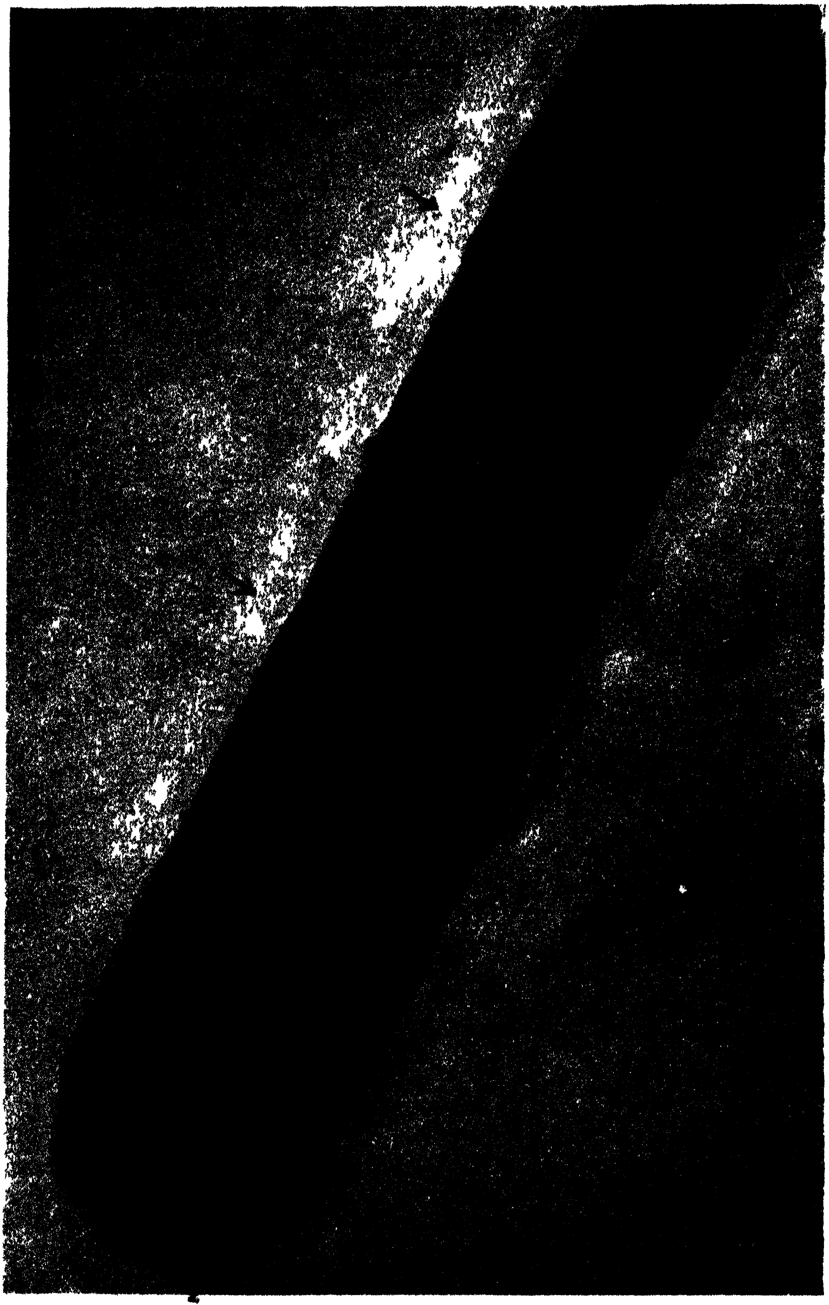
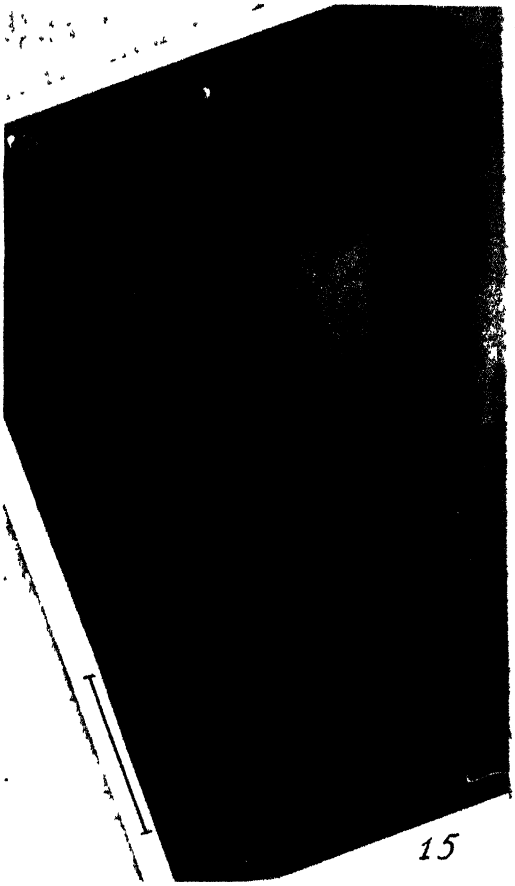


Fig. 14 A cell in the presporal stage Note the many dense bodies with irregular outlines but of a generally circular or elliptical form When marginal, the form and location of these bodies (indicated by arrows) show that they are rather thin and just at the inner side of the cytoplasmic membrane Culture 51½ hours old at 35 C Photographed at 150 kv Length of scale = 1 μ

the more elastic (figures 2 and 4). Both coats are shed during germination (figures 5), and there is no evidence for the presence of an innermost coat which becomes the wall of the germ cell, as stated by Brefeld (1881). However, the wall of the germ cell can be observed as soon as the spore coats are split (figure 6).



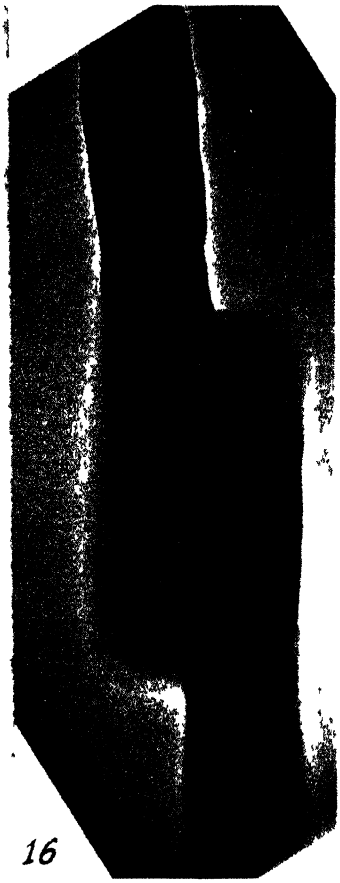


Fig. 15 Cells from a culture, 51½ hours old at 35 C on nutrient agar Cell *a* contains a forespore and one lipoprotein inclusion Cell *b* also contains a barely noticeable forespore. Inclusions are present in all cells Photographed at 150 kv

Fig. 16 Two sporangia each containing one spore Note that the cytoplasm on either side of the spore is of equal density Note slight shrinkage of spore protoplasm Culture 51½ hours old at 35 C Photographed at 150 kv

Length of scales = 1 μ

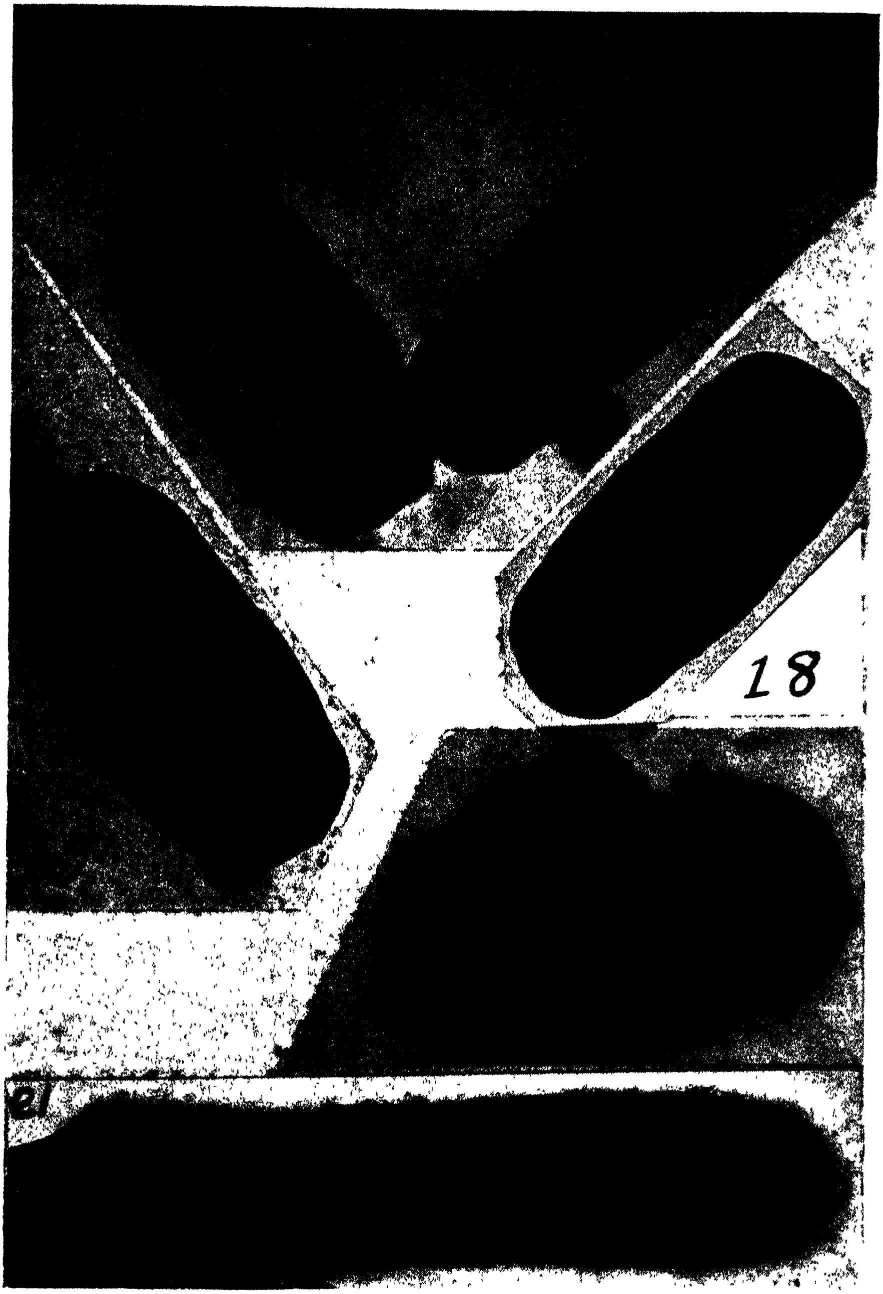
It is possible that it was present but invisible in the resting spore, but more likely that it was formed during germination, as claimed by Preisz (1919).

Germination of the endospore. Germination is initiated by a slight decrease in opacity and by unilateral swelling (figure 7). Rarely, the spore appears to swell all around (figure 7*a*). This is followed by cracking of the inner coat and subsequent tearing of the outer, elastic one at the bulging side (figure 4). Then one end of the germ cell slips out of the split and bent coat (figures 10 and 11). Occasionally, the two halves of the spore coats are completely severed, and each remains as a cap around one end of the germ cell (figure 9). It is not clear whether this is due to failure of the end of the germ cell to slip out of the coat or whether it is characteristic of cases in which the spore swells all around. The cast-off coats remain opaque along the intact periphery, particularly at the poles (figures 12 and 13).

Subsequent development. The germ cell appears homogeneous and does not bear flagella (figure 13). We are not certain whether flagella appear in the second or third generation. After a period of active growth during which the vegetative cells appear homogeneous, one observes in certain cells dark bodies of elliptical to almost circular outlines, 0.1 by 0.12 μ to 0.14 by 0.2 μ. There may be over 20 of these per cell (figure 14). Close examination reveals that the outlines of these bodies are not smooth curves. Since the outline of one of these bodies is a portion of an ellipse at the optical boundary of the cytoplasm, they must be thin and located at the surface of the cytoplasm or possibly in the cytoplasmic membrane (figure 14). Their form, size, and location suggest a relationship with the bodies of type *B* observed by Knaysi and Baker (1947) in the same organism grown in a nitrogen-free medium. Since these bodies are formed after the growth phase and are observed in cells in which sporulation has already begun, they are probably related to or identical with the lipoprotein granules formed, at this cultural stage, in the cytoplasmic membrane and eliminated into the cytoplasm (Knaysi, 1945*b*). This stage is followed by the formation of the forespore (figure 15), which appears homogeneous and develops into the endospore (figure 16). Details of the processes involved in the transition from forespore to endospore were not observed. Inside of its mother cell, the endospore resembles type *a* or *b* described above. Shrinkage of the spore protoplasm, most pronounced at the poles, is probably due to drying. Type *c* is observed only among free spores and is probably the result of incipient germination due to the liberation of food by autolysis, to a fresh supply of food by diffusion from remote parts of the slant, or to a greater availability of food because of an increased oxygen concentration around the spores as the surface film of water is reduced by evaporation. There is no indication that the sporangial protoplasm in the neighborhood of the spore is more opaque to the electrons than is that present in the sterile portion of the sporangium (figure 16).

DISCUSSION

The present investigation has shown, in a striking manner, considerable differences between endospores in their mother culture, and has thus given a morphological basis for the considerable differences, observed by Hodge and Knaysi (1937), between spores of the same culture in their resistance to heat. It has given definite information about the number and certain physical properties of the spore coats, about the details of spore germination, and about the time of appearance of the flagella in the organism investigated. For reasons shown by Knaysi and Baker (1947), it has given no information about the structure of the



FIGS 17 to 19. Various types of spores. Photographed at 50 kv

FIG. 20. A germinating spore. Photographed at 50 kv.

FIG. 21. A young vegetative cell from a culture, 3½ hours old at 35 C, on nutrient agar. Photographed at 50 kv.

Length of scale = 1 μ.

spore protoplasm, or of the protoplasm of the vegetative cell, other than revealing the presence of the lipoprotein inclusions in the presporal stage.

A study of the literature reveals that there has been no agreement on the number and properties of the coats in the resting endospore, or on their behavior during germination. Brefeld (1881) believed in the existence of two coats; the outer one is shed and the inner one becomes the wall of the germ cell. Others (De Bary, 1884; Klein, 1889) wrote of a spore coat surrounded by a weakly refractive slime layer. Meyer (1897, 1899) believed he had demonstrated two coats and a slime layer, all of which are shed. Preisz (1904, 1919) saw one coat and stated that the wall of the germ cell is formed during germination. Lewis (1934) considered a spore coat, possibly in two layers, and an outer, stainable layer, which is a residue of what he called the exosporium. The present investigation shows the existence of only two coats, an inner, somewhat rigid one, and an outer, highly elastic one; the latter is more like the thin "capsule" seen by Lamanna (1940) in certain sporeformers than like the slime layer of De Bary (1884), Klein (1889), and Meyer (1897, 1899). The present investigation finds no evidence for the pre-existence of the wall of the germ cell in the resting spore, and supports the view of Preisz (1919) that such a wall is formed during germination.

The existence of a germ pore has been claimed only for *Bacillus bütschlii* (Schaudinn, 1902). The possibility remained, however, that germ pores are present, but unresolved, in the endospores of smaller organisms. We found no indication of a germ pore in the organism investigated, and everything takes place as if the inner coat is cracked and the outer one torn by a combination of growth pressure and chemical action.

Figures 17 to 21 are taken from a parallel investigation of *Bacillus mycoides* with the EMU. A comparison of these figures with those of similar material taken with the high-voltage microscope shows a definite advantage in the use of high voltage for the study of dense bodies such as the bacterial endospore.

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SUMMARY

The structure of the endospore of *Bacillus mycoides* and its development from spore to spore was studied with the high-voltage RCA electron microscope. The endospores, in their mother culture, vary considerably in appearance and, on that basis, may be classified in three groups.

The spore protoplasm appears homogeneous and is surrounded by two coats. Of the two coats, the inner one is the more rigid and the outer one the more elastic; both are shed upon germination. There is no evidence for the pre-existence, in the resting spore, of a third coat that becomes the wall of the germ cell.

Spore germination is described in detail. The germ cell does not bear flagella and its protoplasm appears homogeneous. The lipoprotein inclusions which

appear in the presporal stage were observed. The forespore appears homogeneous. The sporangial cytoplasm in the neighborhood of the spore is not more opaque to the electrons than that present in the "sterile" portion of the sporangium.

Micrographs taken with the EMU are reproduced to show the advantage of using high voltage in the study of dense bodies such as the bacterial endospore.

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DEMONSTRATION, WITH THE ELECTRON MICROSCOPE, OF A NUCLEUS IN BACILLUS MYCOIDES GROWN IN A NITROGEN-FREE MEDIUM

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Although the bacterial cell has been extensively investigated with the electron microscope, only a few papers have dealt with the problem of the nucleus.

Piekarski (1939) stated that the nucleus like structures or nucleoids demonstrable with the Feulgen reaction and with the ultraviolet microscope in *Eberthella typhosa*, *Escherichia coli*, *Serratia marcescens*, *Proteus*, and *Pseudomonas aeruginosa* may be seen with the electron miscrosope in individual cells of most old cultures, but rarely in cells from young cultures. In old cultures, the cell contains a single, central nucleoid; in young cultures there is a nucleoid at each end of the cell. Piekarski and Ruska (1939) confirmed these conclusions, but found little structural differentiation in the cells of *Sarcina alba*, *Sarcina lutea*, and *Bacillus subtilis*.

Knaysi and Mudd (1943) studied a number of bacteria at accelerating potentials varying from 60 to 200 kv. At 60 kv, the young cells of *Staphylococcus flavo-cyaneus*, *Staphylococcus aureus*, and *Streptococcus pyogenes* appeared homogeneously opaque to the electrons; those of *Neisseria gonorrhoeae* were homogeneous and semitransparent; those of *Brucella abortus* and *Pasteurella pestis* showed a homogeneous, transparent protoplasm containing one to several bright vacuoles. Old cells of the latter two organisms showed a transparent protoplasm containing one or two opaque bodies of unknown nature. Mature cells of *Neisseria meningitidis* had a transparent protoplasm containing one opaque body per cell, this body was insoluble in water at 80 C, indicating that it was not volutin. Treatment of the opaque cells with dilute sodium bicarbonate obscurely revealed the presence of internal bodies in *Staphylococcus flavo-cyaneus*, but not in *Staphylococcus aureus*. With the high-voltage microscope, cells of *S. flavo-cyaneus* from a 2-day-old culture were transparent and contained beautiful, often paired, opaque bodies identified as the nuclei previously demonstrated by Knaysi (1942). Cells from young cultures were either less transparent with poorly defined nuclei or homogeneously opaque. The cells of *Streptococcus pyogenes* became transparent but showed no internal differentiation, although it was reported (Sevag, Smolens, and Lackman, 1940) that they contained relatively large quantities of thymonucleic acid. On the basis of these observations, Knaysi and Mudd concluded that bacteria contain nuclear material which, depending on conditions yet unknown but probably related to environment and development, may be diffuse in the protoplasm or may be partly or totally differentiated into a nucleus.

Baylor, Appleman, Sears, and Clark (1945) studied the internal structure of *Rhizobium leguminosarum* with and without previous treatment with various solvents and nuclear stains. Untreated cells and cells stained with Heidenhain's hematoxylin contained poorly visible opaque bodies. Hydrolysis with HCl, application of the Feulgen technique, or staining by several other procedures caused distortion and increase in the opacity of the protoplasm. Only in cells treated with dilute sodium bicarbonate were the internal bodies clearly visible. These bodies were soluble in acetone and in 2 per cent nitric acid. The authors concluded, however, that they may be nuclei.

The method used in this investigation is based on observations recently made by the senior author. It was shown (Knaysi, 1945*a*) that the endospore of *Bacillus mycoides* contains a relatively considerable quantity of a substance which is an excellent source of nitrogen but not a source of energy. More recently (Knaysi, 1946*c*), it was found that the volutin inclusions of the yeast *Hansenula anomala* are readily utilizable as a source of nitrogen but not for energy. Reinvestigation of germinating spores of *Bacillus mycoides* showed that the source of nitrogen they contain gives microchemical reactions of volutin, which is chiefly ribonucleic acid (Delaporte, 1939). When the spores are heavily planted in a nitrogen-free medium, many germinate; the nucleic acid is gradually used up and soon disappears from the vegetative cells. These cells have a low density, low refringence, and a low affinity for dyes. Fixed by heat and mounted in a film of an aqueous methylene blue solution of pH 3.5 to 4.0, they show deeply stained bodies in a faintly stained cytoplasm, a behavior similar to that of *Hansenula anomala*. When this yeast is grown in a glucose solution and the cells observed as described above, only the nucleus is deeply stained. At pH 3.0, the cells of both remain unstained. Since we have suspected for some time that homogeneous staining of young cells and their homogeneous opacity to the electrons may be due to the same cause, it seemed desirable to find out whether or not cells of usually opaque bacteria that show internal differentiation with the light microscope when grown in a nitrogen-free medium would also show differentiation with the electron microscope.

ORGANISM AND METHOD

We selected, for this study, strain C_2 of *Bacillus mycoides*, on which the preliminary observations reported above were made. At 50 kv, the protoplasm of young cells of this organism grown on nutrient agar appears homogeneously opaque (figure 1); at 150 kv, it becomes homogeneously transparent (figure 2).

The nitrogen-free medium first used had the following composition: glucose, 0.2 g; equimolar mixture of KH_2PO_4 and K_2HPO_4, 0.23 g; distilled water, 100 ml. The spores were harvested from an agar slant culture, washed several times with sterile, distilled water, and resuspended in similar water. Enough of the heavy suspension was inoculated into the nitrogen-free medium to produce a definite haze. After various periods of incubation, the cells were centrifuged out, resuspended in distilled water, mounted on collodion, and examined in the usual manner with the high-voltage microscope. Cells thus obtained were highly vacuoled,

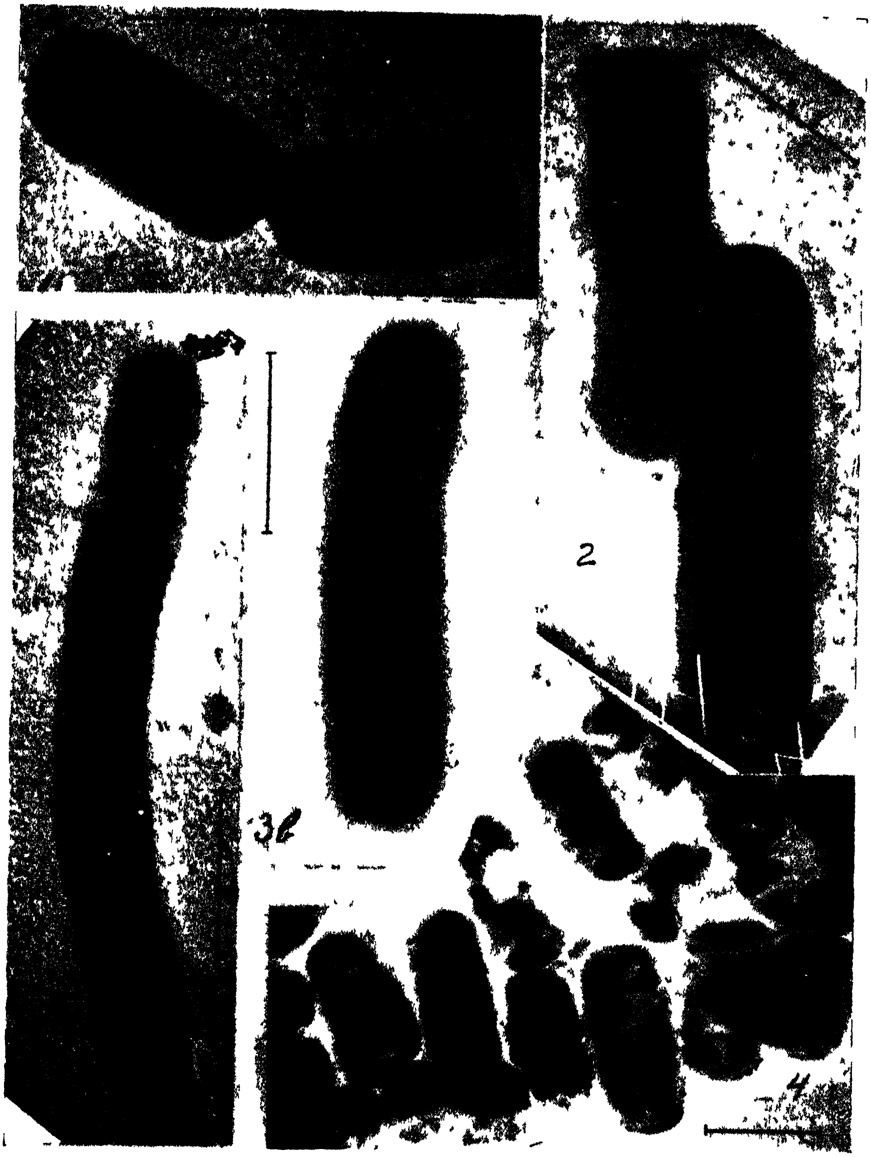


FIG. 1 Sister cells from a nutrient agar slant, culture 1½ hours old at 35 C, 50 kv × 23,500

FIG. 2 Cells grown on a nutrient agar slant culture 51½ hours old at 37 C, 150 kv × 26,000

FIG. 3 Cells from a 17 hour old culture in the glucose phosphate medium, at room temperature Note the many vacuoles and the several nuclei In *a* the arrow points to a dividing nucleus In *b* note also the dense slime layer the cell wall and the cytoplasmic membrane Both cells photographed at 150 kv × 25 000

FIG. 4 Cells grown on a nutrient agar slant, culture 5 hours old at 37 C Smear was heat fixed and treated with a 0 5 per cent solution of sodium bicarbonate for 90 minutes at room temperature, 50 kv × 15,000

Length of scales = 1 μ

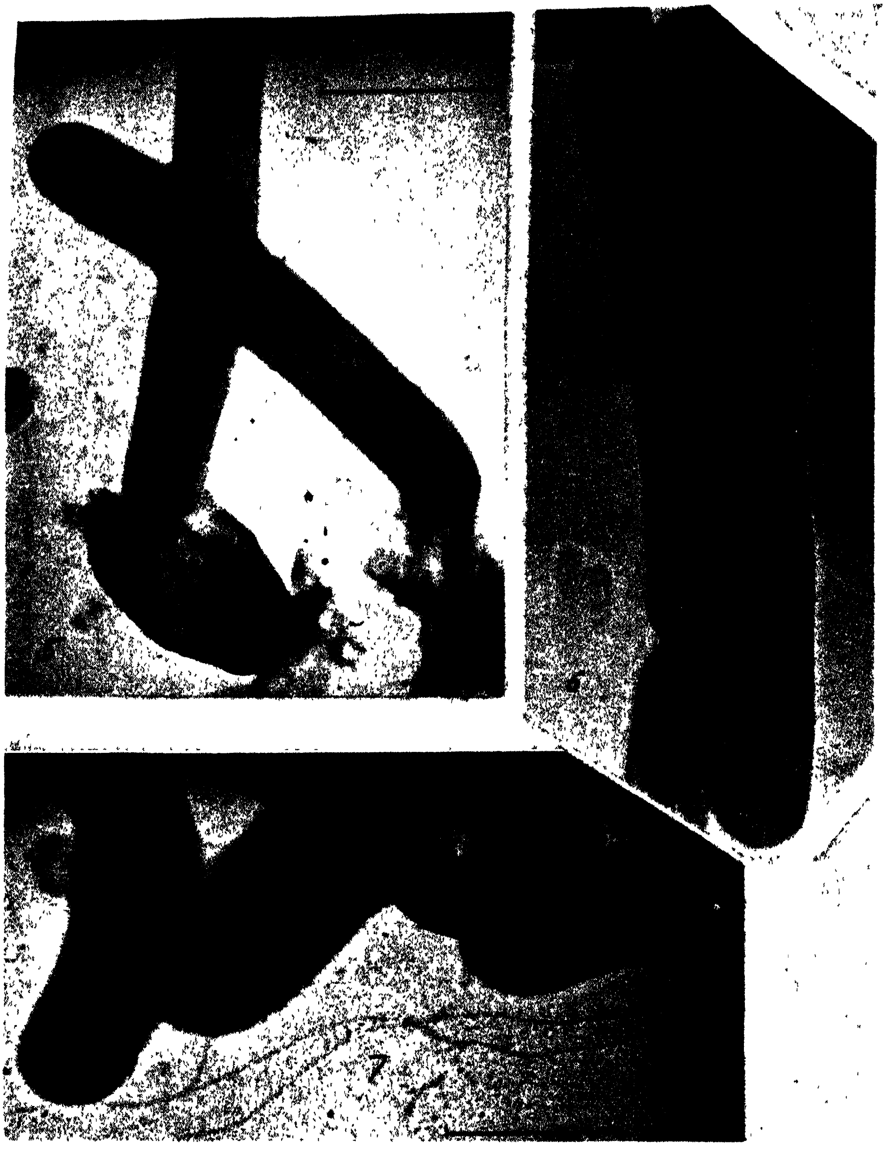


FIG. 5. Two germ cells from a 16-hour-old culture, at room temperature, in the glucose-acetate medium. Note the transparent cytoplasm and the two types of bodies, 50 kv. × 23,500.

FIG. 6. Vegetative cells showing evidence of growth. Culture, voltage, and magnification as for figure 5.

FIG. 7. Vegetative cells one of which appears to bear flagella. Note also two other flagella. Culture, voltage, and magnification as for figure 5. Note also nuclei and bodies of type *B*; the structure of the latter can be clearly seen in the two extreme cells.

Length of scales = 1 μ.

encapsulated, and contained each 5 or 6 opaque bodies sometimes showing evidence of division (figure 3). On the other hand, the cells were not readily centrifuged out and often became opaque again, resynthesizing nucleic acid from the glucose, the phosphate, and nitrogen probably liberated by autolysis. This was avoided by substituting sodium acetate for potassium phosphate in the medium. This medium has an initial pH of about 7.4, and the pH remains in the neighborhood of 6.8 even several days after inoculation. It is buffered dynamically; the acids produced, chiefly from the glucose, are continuously neutralized by the NaOH formed when the acetate radical is utilized. In this medium, the cells grow normally, free of vacuoles and slime, and many complete the life cycle; their protoplasm is so transparent that they have to be observed at 50 or 30 kv (figures 5 to 20).

OBSERVATIONS AND CONCLUSIONS

In the glucose-acetate medium many of the spores germinate (figure 5), and germination is followed by growth of the vegetative cells (figure 6); these may be motile (observation with the light microscope) and bear flagella (figure 7); they contain two types of internal bodies (figure 8) distinct from each other by the degree of their opacity, their size, apparent form, and their behavior. Both types are present at all stages of development (figures 5, 8, and 9).

Type A consists of large, highly opaque bodies, 0.22 by 0.29 μ to 0.43 by 0.50 μ. There may be 1 to 6 of these in a cell. A body of this type sometimes seems to be surrounded by a membrane which may be an artifact due to drying, but there is no sign of internal differentiation (figures 10 and 11). Evidence of division is frequent, but the manner in which division takes place is not yet clear; sometimes one observes two bodies in close proximity separated by a dark streak as broad as the bodies themselves and resembling nuclei in early telophase (figures 8, 11, and 12); at other times it appears as if a body, lying in the middle of a cell, has been split in two by division of the cell, although it may be possible that division of the body preceded that of the cell (figures 14 and 15). We never saw what may be considered as *clear* evidence of division by simple constriction. Further study is necessary before the mechanism of division can be definitely described.

When the cell is ready to form a spore, one observes a faintly delimited forespore with these bodies grouped usually at each of its poles (figures 9, 13, and 16); occasionally, one or more bodies may be located elsewhere (figures 17, 18, and 19). In a sporangium that contained only two of these bodies, one is located at each pole of the forespore (figures 9 and 13). One rarely observes a body of this type, originally present in the mother cell, that does not become enclosed in the forespore. Almost always, the forespore seems to appear in its full size and characteristic form. Nevertheless, isolated observations (figures 13 and 20) seem to suggest the following possible sequence: Two bodies, or groups of bodies, move to a definite distance from each other; an area, slightly denser than the cytoplasm of the mother cell, appears to grow from each body, or group of bodies, toward the other; the forespore results from the merging of these two areas. We never recognized a forespore containing only one body. The length of the forespore is determined by the relative position of the two bodies, or groups of bodies, which seem to produce it, and this is not changed by subsequent development. However, the

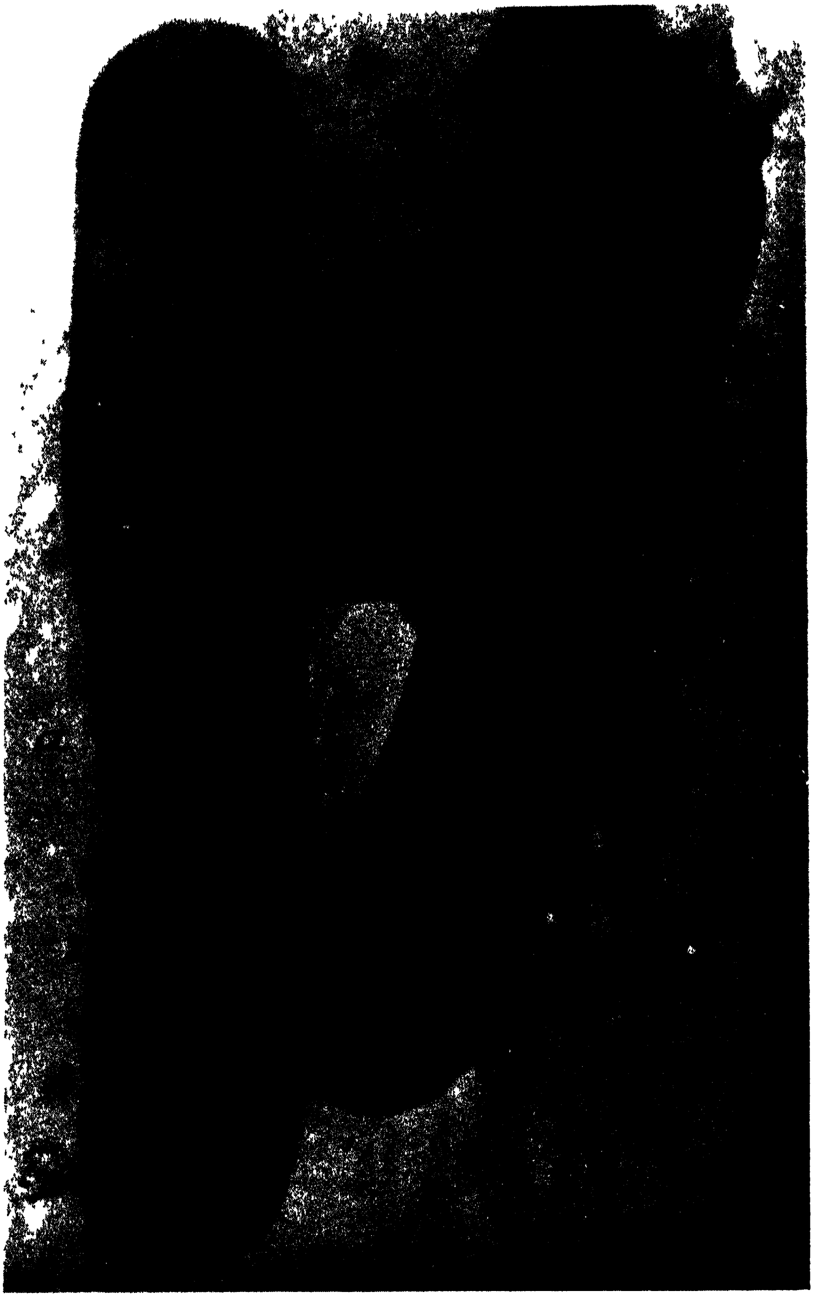
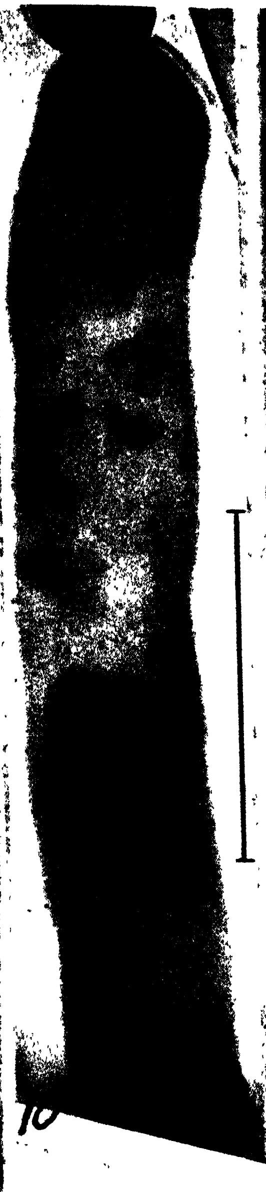


FIG. 8 Vegetative cells from a culture in the glucose acetate medium, 16 hours old at room temperature At the lower end of cell *a*, there are two nuclei separated by a broad, dark zone, the whole resembling nuclei in early telophase Note absence of constriction. All cells show numerous bodies of type *B*, the structure of which is best observed with a hand lens. A few of these appear free in the background and probably originated from autolyzed cells Photographed at 50 kv. × 48,000.

Length of scale = 1 μ.



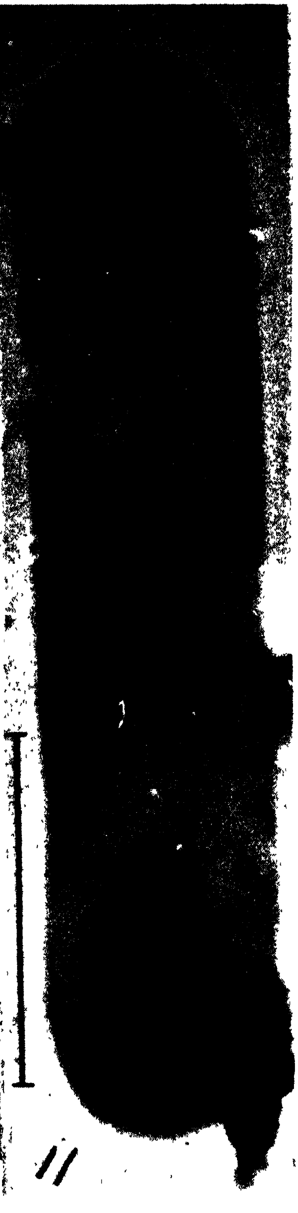


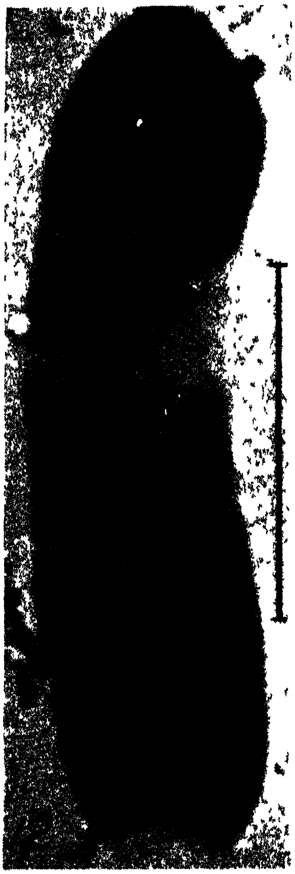
FIG. 9. Sister cells from a culture, two days old at room temperature, in the glucose-acetate medium. Note a young forespore in each cell, the position of the nuclei in the forespore, and the presence of bodies of type *B*. The bright spot in the upper cell is the image of the filament. Note the semicircular fringes of the split cross plate. Photographed at 50 kv. × 50,000.

FIG. 10. Cell from a culture, 16 hours old at room temperature, in the glucose-acetate medium. Note possible evidence of a membrane around the nucleus. Photographed at 50 kv. × 48,000.

FIG. 11. Culture as for figure 9. Upper end shows two neighboring nuclei joined by a broad, dark zone; the inner nucleus shows possible evidence of a membrane. Photographed at 50 kv. × 48,000.

Length of scales = 1 μ.

density of the forespore increases gradually. Mature spores were occasionally found in the medium used (figure 21), but the steps in the transition from a forespore to a spore were not observed.



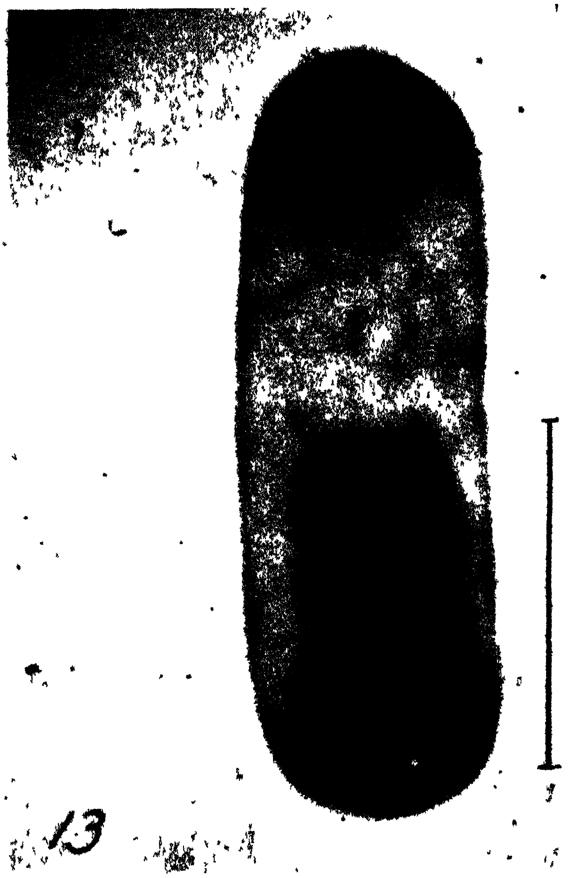


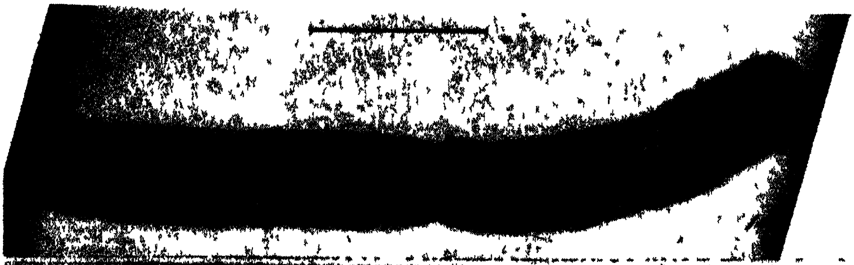
FIG. 12 A cell from a culture, 2 days old at room temperature, in the glucose-acetate medium Lower end contains what appears to be a dividing nucleus, 50 kv. × 18,000

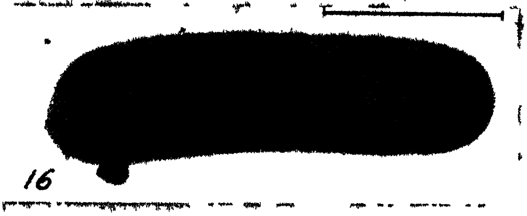
FIG. 13 A flagellum bearing cell Culture 1 day + 16 hours old at room temperature in the glucose acetate medium. Note the young forespore with a clue to its mode of formation, an earlier stage may be seen in figure 20. Note also position of the nuclei in the forespore Photographed at 50 kv. × 47,000

Length of scales = 1 μ.

In view of the fact that the function of a body is determined by its behavior, the observed behavior of these bodies of type *A* during the life cycle of *Bacillus mycoides* identifies them as the nuclei of the cell.

Type B (figures 7 and 8) consists of bodies, a few to about 40 per cell, smaller than the nuclei (0.08 by 0.1 μ to 0.13 by 0.17 μ) and much less opaque. Careful examination of many micrographs shows that they are extremely thin, each with a circular or elliptical outline consisting of a beaded thread, 40 to 60 A in thickness, and a central area which sometimes appears homogeneous but more





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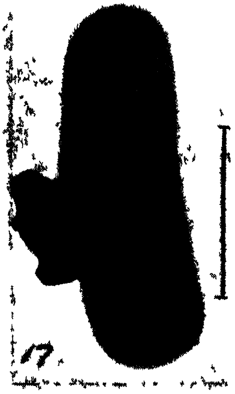

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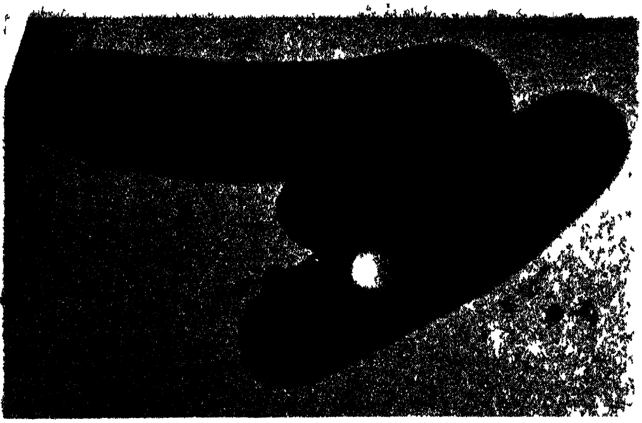


Fig. 14 Sister cells Culture 1 day + 16 hours old at room temperature in the glucose acetate medium Note what appears to be division of the cell through the median nucleus Photographed at 50 kv × 23 500

Fig. 15 Sister cells Culture 2 days old at room temperature in the glucose acetate medium Note what appears to be simultaneous division of cell and nucleus Photographed at 50 kv × 23,500

Fig. 16 A cell with a young forespore Culture 2 days old at room temperature in the glucose acetate medium Note groups of nuclei and their polar positions in the forespore. Photographed at 50 kv × 23 500

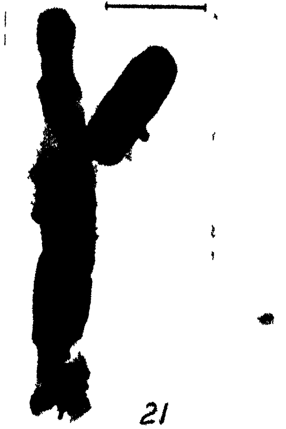
Fig. 17 Cell showing a forespore with a lateral nucleus Culture voltage and magnification as for figure 16

Fig. 18 Cell with a forespore containing 5 nuclei 2 of which are polar Culture 1 day old at room temperature in the glucose acetate medium Photographed at 50 kv × 24 000

Fig. 19 Two cells each containing a forespore In *a* the forespore almost fills the cells. Culture 2 days old at room temperature in the glucose acetate medium Photographed at 50 kv × 21,000

Length of scales = 1 μ

often consists of minute granules and threads in a wide variety of patterns. The central area is often separated from the boundary line by a transparent ring, and may be surrounded by a second boundary line similar to the outer one and concentric with it. These bodies appear to be part of a background also consisting of a network of granules and beaded threads similar in dimensions but of much lower opacity to the electrons. Like the spots of a leopard, they are groups of dark elements in an otherwise similar background. Because of the large depth of focus of the electron microscope, it is not possible to determine directly the location of these bodies in the cell. However, since they are present in autolyzed cells (figures 22 and 23c), and are semielliptical along the margin of the cytoplasm (figure 23), it may be concluded that they are in the cytoplasmic membrane. They do not visibly participate in the formation of the forespore as do the nuclei, nor do they move away from the foresporal area (figure 9) as do the lipoprotein inclusions usually liberated into the cytoplasm before sporulation (Knaysi, 1945b, 1946b). Further discussion of these bodies will be found in the following section.



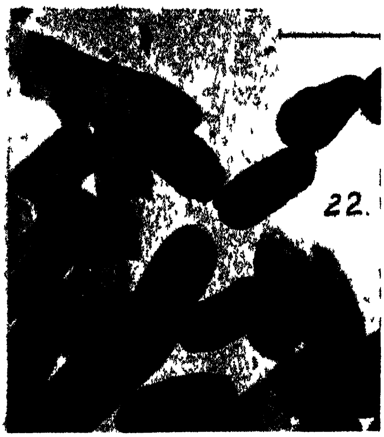


FIG. 20. Cell showing two groups of nuclei and the beginning of forespore formation. Note the semiellipsoidal dense region to the right of the pair of nuclei, a more advanced stage can be seen in figure 13. Culture 16 hours old at room temperature. Photographed at 50 kv. × 23,500.

FIG. 21. A sporangium containing a mature endospore. Culture 4 days old at room temperature in the glucose acetate medium. Photographed at 50 kv. × 13 000.

FIG. 22. Culture, voltage and magnification as for figure 21. Note ghost cells still showing bodies of type *B*. Photographed at 50 kv. × 13 000.

Length of scales = 1 μ.

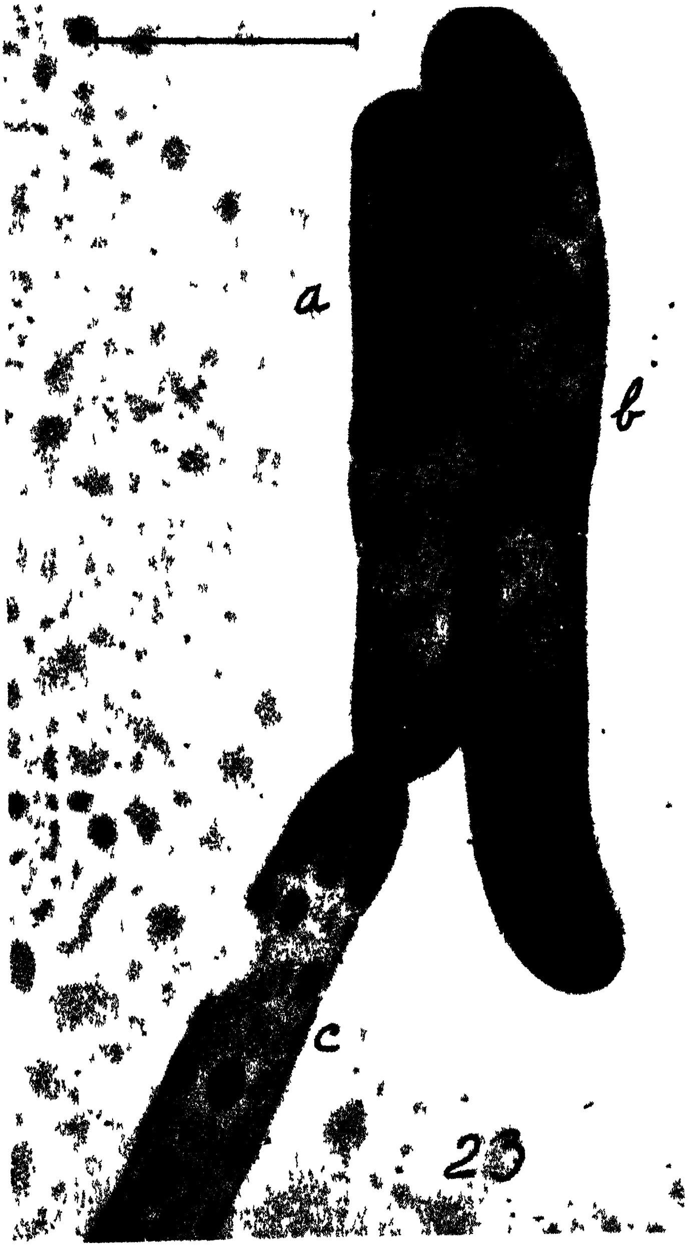


Fig. 23. Two normal vegetative cells (*a* and *b*) and one ghost cell (*c*), all showing marginal bodies of type *B* with semielliptical outlines, indicating that they are thin and present in the cytoplasmic membrane. Culture 16 hours old in the glucose acetate medium. Photographed at 50 kv. × 35,500.

Length of scale = 1 μ.

DISCUSSION

The observations reported in the previous section prove, we believe, the existence of one to several nuclei in the cell of *Bacillus mycoides*. The evidence does not rest solely on similarity in staining reactions, of doubtful specificity, between the bodies observed and the nuclei of more highly developed organisms, but on the behavior of these bodies during the life cycle of the organism, which is the only sure criterion of nuclear function: constancy of presence, evidence of division, and a leading role in the formation of the endospore. Since the only treatment the cells received was quick drying, it cannot be said that the observed bodies may have been coagulated nuclear material or other constituents of the protoplasm. Since they are readily demonstrable with the light microscope, it cannot be said that they were formed by the action of the electrons. We are aware, however, that the novelty of the procedure may raise certain questions regarding the viability of the cells investigated, the genuineness of the forespores, the possibility that the bodies we observed are mere inclusions or, granting that they are nuclei, that they would also be present in cells grown on ordinary culture media.

The first question can be answered by stating that cells similar to those observed were able to grow in microcultures and, therefore, were viable. It can also be answered by pointing out that dead cells do not form endospores, or by *reductio ad absurdum*, since our inoculum consisted entirely of free spores which germinated (figure 5) and grew (figure 6) and formed endospores in the medium used (figure 21). The only vegetative cells found in the cultures at various stages of their development were transparent and contained the bodies we recognized as nuclei: some of the cells bore flagella (figure 7), and motility was observed with the light microscope. Therefore, it would be absurd to assume that all the cells we observed were dead and partially autolyzed.

We believe that we observed genuine forespores because, in a medium like the one used, vacuoles would be more transparent than the cytoplasm; their number per cell and their size and form would vary considerably. Furthermore, in a medium which induces vacuolation, the presence of vacuoles would have been more general, as in the glucose-phosphate medium (figure 3), and would not have been restricted to a relatively few cells. The forespores are denser than the cytoplasm of the mother cell; only one per cell was observed, which is the case in nonfilamentous growth of *Bacillus mycoides*; their size and shape also correspond to what would be expected in this organism. In one case we observed a forespore which almost filled the mother cell (figure 19), a condition we often observed in this and several other members of the genus *Bacillus*.

The nuclei are not inclusions. They take nuclear stains in the conventional manner. They are distinct from volutin by their relatively high isoelectric point and their insolubility in 0.04 per cent sodium bicarbonate in 90 minutes at room temperature; they are distinct from lipoid inclusions by their relatively low refringence, their presence in the germ cells and subsequent early generations, and their unstainability with Sudan black B. Furthermore, they are not to be considered a new type of inclusions, since inclusions would not occupy a definite position in a vacuole or in a forespore and, when several inclusions are present, some would remain free in the cytoplasm of the mother cell, a situation never clearly recognized during the present investigation. It must be added that, with the light microscope, the forespore appears homogeneous in the living cell (Knaysi, 1946*b*), as well as in cells stained to show inclusions. There are certainly no lipoid granules, and the ribonucleic acid the forespore contains is diffuse throughout the cytoplasm.

The question as to whether or not nuclei are present in cells grown on ordinary media is more difficult to answer, since cells grown on ordinary media show no internal differentiation. It may be remembered, however, that cells treated with 0.5 per cent $NaHCO_3$ suggest the existence of bodies which resemble the nuclei observed in transparent cells (figure 4). More definite information is obtained when a culture in the glucose acetate medium is examined with the light microscope. In such a culture one observes vegetative cells, numerous refractile spores, and many nonrefractile spores which used up their reserve material but failed to germinate. When these are mounted in a drop of methylene blue solution of pH 3.5 to 4.0, they show a faintly stained cytoplasm and two deeply staining bodies, one at each pole of a spore, which have a considerable resemblance in size and form to the nuclei observed with the electron microscope. Since these spores were formed on ordinary media, it seems logical to conclude that nuclei similar to those observed in the forespores of transparent cells are also present when the spores are formed on ordinary media.

Other questions may also be asked. For instance, does the nucleus contain more than one chromosome? Are the results now obtained with *Bacillus mycoides*, and those previously obtained by Knaysi and Mudd (1943) with *Staphylococcus flavo-cyaneus*, applicable to other bacteria? What is the biological nature of the endospore?

The correct answers to these questions will have to await further research. It is safe to predict, however, that answers will soon be forthcoming, since we now have a procedure which, directly applied or modified, makes investigation of the internal structure of bacteria with the electron microscope possible, thus eliminating much of the uncertainty which, in the past, has characterized such investigations.

The nature and function of the bodies of type *B* are not definitively established. They are part of the cytoplasmic membrane and, except when they appear homogeneous, they cannot be structurally differentiated from the rest of that membrane. Both seem to consist chiefly of a network of beaded threads and granules, probably representing complex molecules. Their only distinguishing feature is the greater opacity of their threads and granules, probably acquired through synthetic activity. Their general appearance is that of dark spots in an otherwise similar background. They bear close resemblance in form and dimensions to the dark disks observed during the presporal stage of the same organism by Knaysi, Baker, and Hillier (1947). It is not unlikely that the bodies of type *B*, which are present throughout the life cycle, become gradually more opaque by continuous synthesis, and that they become visible in the presporal stage even in cells grown on ordinary media. It may be recalled that the formation of lipoprotein granules in the cytoplasmic membrane of *Bacillus mycoides* and *Bacillus cereus* and elimination of these granules into the cytoplasm were observed by Knaysi (1945*b*, 1946*a*). Thus the bodies of type *B* may be the primordia of the presporal inclusions. Their chief role, however, may be the formation of cross plates at the time of cell division, a function which is suggested by the observations of Knaysi, by a frequently linear arrangement of these bodies across the cell (figure 7), and by the occasional presence of cross plates the borders of which seem to consist of consecutive semielliptical fringes (figure 9).

A relation between ribonucleic acid and the gram reaction was found by Dubos and MacLeod (1938) and by Henry and Stacey (1943). However, the function of ribonucleic acid in the bacterial cell, its correct location, and its causal relation to uniform staining and uniform opacity to electrons were recognized, for the first time, in the present investigation. This led to a procedure by which ribonucleic acid is removed, and its reformation is prevented, without injury to the cell or interference with its normal development. Work now in progress indicates that application of this procedure transcends the limits of the gram reaction.

It has been pointed out in a previous section that many spores fail to germinate in the medium we used; this is in no way contrary to the expected behavior of endospores. However, since a high percentage of germination would be desirable, we are now investigating the problem and the results will be the subject of a future communication. It suffices now to state that when the spore suspension is obtained from a week-old slant culture on a medium made up of meat infusion (half strength), 100 ml + tryptone, 0.5 g + glucose, 0.5 g + agar, 1.5 g, and when the inoculum is such that it amounts to 5 or 6 million spores per ml of the glucose-acetate medium, a culture one day old at 35 C may consist of 30 to 50 per cent of vegetative cells. To obtain such results, however, it is necessary that the spore suspension be concentrated and, preferably, less than 2 weeks old.

ACKNOWLEDGMENTS

The senior author is deeply grateful to Doctor Stuart Mudd who, besides being instrumental in securing for him the opportunity to conduct his share in this investigation, has given him ample laboratory quarters and facilities while this work was in progress.

SUMMARY

When the endospores of *Bacillus mycoides*, C_2, are washed with sterile, distilled water and heavily inoculated into a nitrogen-free medium, many germinate and gradually use up their supply of ribonucleic acid. The germ cells and subsequent generations of vegetative cells are transparent to electrons even at 50 or 30 kv and show two types of opaque bodies.

Bodies of the first type are relatively large and highly opaque; they show evidence of division and are totally enclosed in the forespore; they do not seem to divide by simple constriction. These are the nuclei of the cell.

The bodies of the second type are smaller and very thin; they consist of beaded threads and granules located in the cytoplasmic membrane; they do not, visibly, take part in the formation of the endospore. There is evidence that they are endowed with synthetic power and that they are involved in the formation of cross plates at the time of cell division.

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INHIBITION OF METHIONINE SYNTHESIS IN ESCHERICHIA COLI BY 2-CHLORO-4-AMINOBENZOIC ACID AND SULFANILAMIDE

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The bacteriostatic action of 2-chloro-4-aminobenzoic acid (2-Cl-PAB) was found by Wyss, Rubin, and Strandskov (1943) to be completely reversed by *p*-aminobenzoic acid. This, according to the theory of Woods (1940) that *p*-aminobenzoic acid is a specific antagonist for sulfanilamide, classified the bacteriostatic action of 2-Cl-PAB as sulfonamide activity. The bacteriostatic action of this compound did, however, differ from that of the sulfonamides in that it could be completely reversed by methionine. The action of the sulfonamides is only partially reversed by this amino acid. Shive and Roberts (1946) have suggested that the sulfonamides interfere with a number of enzyme systems in which *p*-aminobenzoic acid functions and that the enzyme system which is most sensitive to the sulfonamides is the one responsible for the synthesis of methionine. Because the bacteriostatic action of 2-Cl-PAB is completely reversed by methionine, they postulate that 2-Cl-PAB inhibits only the enzyme system involved in the synthesis of this amino acid.

The work reported here is a study of the inhibition of methionine synthesis by 2-Cl-PAB and sulfanilamide. Strains of *Escherichia coli* made resistant to 2-Cl-PAB and a strain made resistant to sulfanilamide were employed. The mechanism of the development of resistance to 2-Cl-PAB was also investigated.

METHODS

Stock solutions of the compounds to be tested were prepared in sterile distilled water and sterilized by minimal heating. The desired quantities were added to each test tube and the volume was made up to 2 ml with sterile distilled water. A glucose, inorganic salts, asparagine medium, adjusted to pH 7.0, was inoculated with the strain of *E. coli* to be tested, and 8 ml of the inoculated medium were added to each of the tubes containing 2 ml of the drug solution, as well as to controls containing 2 ml of sterile distilled water. The inoculum was prepared by growing the test organism in the synthetic medium at 37 C for 24 hours. The cells were washed twice with saline and diluted so that the inoculated medium contained approximately 1,000 cells per ml. The test solutions were incubated at 37 C, and turbidity readings were made at selected time intervals.

EXPERIMENTAL

We intended to develop resistance to 2-Cl-PAB in *E. coli* by the technique employed in developing resistance to sulfanilamide. This involves continuous transfers in growth-limiting concentrations. We found, however, that complete resistance to 2-Cl-PAB was developed in a single transfer of the organisms in a medium containing inhibitory concentrations of the drug. This is shown by the data in tables 1 and 2. The data in table 1 show that after the 24-hour incubation period no turbidity had developed in the tubes containing 1.0 mg per cent or more of 2-Cl-PAB. After 72 hours, however, the growth in the tubes containing as high as 25 mg per cent of the drug approximated that in the control.

TABLE 1

The bacteriostatic action of 2-Cl-PAB on E. coli

TUBE NO.	2-Cl-PAB	TURBIDITY AFTER 24 hours	TURBIDITY AFTER 72 hours
	mg %		
1	0	0 342	0.342
2	0.2	0.337	0.377
3	0.5	0.2924	0.372
4	1.0	0	0.382
5	2.0	0	0.310
6	5.0	0	0.305
7	10	0	0.387
8	25	0	0.259

TABLE 2

The resistance to 2-Cl-PAB of strains of E. coli previously grown in 0, 5, and 25 mg per cent of the drug

CULTURE NO	2-Cl-PAB	24-HOUR TURBIDITY
	mg %	
1	0	0.328
1	0.2	0.319
1	0.5	0.036
1	1.0	0
6	0	0.276
6	2.0	0.276
6	5.0	0.102
6	10	0.082
6	25	0.046
6	50	0
8	0	0.310
8	5.0	0.305
8	10	0.276
8	25	0.260
8	50	0.194

The resistance of these cultures to 2-Cl-PAB was tested by using cultures prepared from tubes 1, 6, and 8 (table 1) as inocula for the experiment reported in table 2. The data show that the organisms had developed a resistance to the drug and that the degree of resistance of the culture in its first 24 hours of growth is governed by the concentration of the drug in which it was originally grown. The relationship of the degree of resistance to the concentration of the drug used in developing resistance has previously been observed for the sulfonamides by Kirby and Rantz (1943). Organisms isolated from the other concentrations of 2-Cl-PAB, reported in table 1, were also tested, and the same relationship was observed. This resistance to the drug was retained through one year of culturing on nutrient agar.

The relationship of 2-Cl-PAB resistance to sulfanilamide resistance in *E. coli* is shown in table 3. The sulfanilamide-resistant organism is a strain of the *E. coli* culture used in these experiments that was made resistant to sulfanilamide several years ago in this laboratory by continuous transfer in growth-limiting concentrations of the drug. The 24-hour turbidity readings show that the ratio of the lowest bacteriostatic concentrations of 2-Cl-PAB to sulfanilamide is the same for the sulfanilamide-resistant strain as it is for the parent strain. The 2-Cl-PAB-resistant organism was, however, found to be slightly but consistently more sensitive to sulfanilamide than was the parent strain. This shows that resistance to 2-Cl-PAB is not a true sulfonamide resistance, as it has been previously demonstrated by Wyss, Strandskov, and Schmelkes (1942) that organisms made resistant to bacteriostatic concentrations of sulfanilamide are equally resistant to similar bacteriostatic concentrations of the other sulfonamides that are antagonized by *p*-aminobenzoic acid.

TABLE 3

The resistance of a parent strain (1), a sulfonamide-resistant strain (133), and a 2-Cl-PAB-resistant strain (8) of E. coli to sulfanilamide and 2-Cl-PAB

CULTURE NO.	2-Cl-PAB	SULFANILAMIDE	24-HOUR TURBIDITY
	mg %	*mg %*	
1	0		0.328
1	0.2		0.336
1	0.5		0
8	0		0.305
8	25		0.310
8	50		0
133	0		0.301
133	0.5		0.292
133	1.0		0.102
133	2.0		0.082
133	5.0		0
1		2.0	0.337
1		5.0	0.201
1		10	0
8		2.0	0.319
8		5.0	0.027
8		10	0
133		50	0.347
133		100	0

If the inhibition of methionine synthesis is the only function of 2-Cl-PAB and if this inhibition is identical with sulfonamide inhibition of this synthesis, one would expect a 2-Cl-PAB-resistant organism to behave in the presence of sulfanilamide as though methionine had been added to the medium. The data in table 4 show, however, that the concentration of sulfanilamide required to reduce the rate of growth to half-maximum was increased by the addition of methionine for the 2-Cl-PAB-resistant strain as well as for the parent strain. This shows that although the organism has been trained to grow in the presence of this drug, which is completely antagonized by methionine, it cannot synthesize methionine in the presence of sulfanilamide. Sulfanilamide must, therefore, inhibit a process in the synthesis of methionine not affected by 2-Cl-PAB.

TABLE 4

The effect of methionine on the bacteriostatic action of sulfanilamide on a parent and 2-Cl-PAB-resistant strain of E. coli

ORGANISM	METHIONINE	CONCENTRATION OF SULFANILAMIDE GIVING ½ MAXIMUM RATE OF GROWTH
	mg %	*mg %*
1	0	3.8
1	1.0	5.8
8	0	1.9
8	1.0	3.7

1 = parent strain.
8 = 2-Cl-PAB-resistant strain.

DISCUSSION

That the bacteriostatic action of 2-Cl-PAB is closely related to true sulfonamide bacteriostasis is emphasized by the relative resistance of the parent and sulfanilamide-resistant organisms to the drug. This is also added evidence that the inhibition of methionine synthesis is a fundamental function of the sulfonamides. The lack of resistance to sulfanilamide in the 2-Cl-PAB-resistant strain, as well as the increased resistance to sulfanilamide observed in the presence of methionine, shows, however, that sulfanilamide, in addition to inhibiting methionine synthesis in the same manner as does 2-Cl-PAB, exerts an additional inhibitory effect on the synthesis of this amino acid.

The ease with which resistance to 2-Cl-PAB is developed in *E. coli* is unusual. Resistance to the sulfonamides in this organism is developed only after a series of transfers in growth-limiting concentrations. The fact that 2-Cl-PAB inhibits only the synthesis of methionine, whereas sulfanilamide interferes with the synthesis of additional products, could account for this difference.

The rapid development of resistance to 2-Cl-PAB indicates that either a very small percentage of the inoculum was originally resistant to the drug, and that the delayed growth as compared with that in the control was due to the extremely small inoculum, or that all the cells are able slowly to adapt themselves, through some alteration in their growth mechanism, to growing in concentrations of the drug that were originally inhibitory. To determine the mechanism involved, the parent strain was plated on a series of synthetic medium agar plates containing increasing concentrations of 2-Cl-PAB. We found that after 24 hours' incubation the plates containing as high as 0.5 mg per cent of the drug supported growth of a number of colonies equal to the control. No colonies were visible on the plates containing higher drug concentrations. After 96 hours, the plate containing 1.0 mg per cent 2-Cl-PAB contained 25 per cent as many colonies as the control, and each of those having higher concentrations, up to 25 mg per cent, supported growth of one-tenth the number of colonies which grew on the control. Continued incubation for 2 weeks gave no increase in the number of colonies on any of the plates. Organisms isolated from these plates were resistant to the drug, and the degree of resistance was again dependent on the concentration to which they had been exposed. This shows that approximately one-tenth of the cells in the parent culture can develop a resistance to inhibitory concentrations of 2-Cl-PAB and that apparently none of the cells were originally resistant to this drug.

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CONCLUSIONS

Permanent resistance to 2-Cl-PAB in *Escherichia coli* is developed during the first 72-hour growth period in the presence of bacteriostatic concentrations of the drug. The degree of resistance obtained depends on the concentration of drug in which the organisms are grown. Resistance to 2-Cl-PAB is not a property of a small percentage of the original inoculum but is developed in approximately 10 per cent of the cells. This is not a general sulfonamide resistance. Sulfonamide-resistant *E. coli* cells do, however, show a parallel increase in resistance to 2-Cl-PAB. Sulfanilamide appears to inhibit a process in the synthesis of methionine additional to that inhibited by 2-Cl-PAB.

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THE INACTIVATION OF THE V FACTOR BY ERYTHROCYTES AND THE V-SPARING PROPERTY OF NICOTINAMIDE[1]

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The studies described in this report were stimulated by the observation that although "chocolate" agar gave good growth of *Hemophilus influenzae*, the addition of unheated blood or erythrocytes to the chocolate agar resulted in a marked inhibition of the growth of this organism. They deal with the action of human blood on the V factor and with the V-sparing property of nicotinamide.

The destruction of the V factor by a heat-labile system in the fresh serum of rabbits, rats, sheep, and guinea pigs was reported by Knorr (1924). Pigeon and human sera did not possess this destructive property. Knorr was able to inhibit the growth of *H. influenzae* on Levinthal agar by adding sheep blood or washed sheep erythrocytes. Better growth of *H. influenzae* was observed in agar containing very small amounts of intact or lysed sheep erythrocytes than in agar containing greater concentrations. The suggestion was made that an excess of the X or V factors decreased the amount of growth of this organism. Lwoff and Lwoff (1937) reported the destruction of the V factor by a heat-labile substance in the fresh serum of certain animals, and Kohn and Klein (1940) demonstrated the presence of a similar system in human erythrocytes.

Lwoff and Lwoff (1937) showed that purified codehydrogenases (pyridine nucleotide phosphates) satisfied the requirements of *H. influenzae* and *Hemophilus parainfluenzae* for the V factor. Yeast and muscle adenylic acid, nicotinic acid, its amide, and diethylamide were inactive. Schlenk and Gingrich (1942) tested the structural units (nicotinamide, *d*-ribose, and adenylic acid) of the coenzyme molecule individually and together, as well as other nicotinamide-containing nutrilites. Nicotinamide nucleoside supported the growth of these organisms, but the structural units of the coenzyme molecule were inactive. Mann and Quastel (1941) studied the inactivation of partially purified coenzyme derived from yeast by a heat-labile system ("coenzyme nucleotidase") in rat brain and reported the blocking of this inactivation by nicotinamide.

The inhibition of the growth of *H. influenzae* which resulted from the addition of blood or erythrocytes to chocolate agar was found to be due to the removal of the V factor by a heat-labile system present in the erythrocyte. This system was found to destroy the V factor of human erythrocytes or Lwoff and Lwoff yeast extract and, like the "coenzyme nucleotidase" described by Mann and Quastel, could be blocked by nicotinamide.

The destruction of the V factor of erythrocytes and the protective action of nicotinamide were demonstrated in an experiment in which 10 per cent of packed human erythrocytes were mixed and incubated with the clear supernatant of chocolate broth. The chocolate broth was prepared by heating 10 per cent human erythrocytes in Difco tryptose phosphate broth at 85 C for 5 minutes and removing the coagulum by centrifuging. This broth, containing both X and V factors, was designated "XV" broth. Broth containing only the heat-stable X factor was prepared by adding 5 per cent blood to broth, autoclaving at 15 pounds' steam pressure for 15 minutes, and clearing by centrifuging. To portions of these broths, 0.1 per cent nicotinamide was added, and then the effect of the addition of 10 per cent washed human erythrocytes was determined. Five-ml quantities of the mixtures in 50-ml Erlenmeyer flasks were incubated at 37 C for 4 hours, with frequent gentle shaking. The cells were then separated by centrifuging and decanting the supernatant. The supernatant was divided into two equal quantities, one of which was heated at 85 to 90 C for 5 minutes. Five ml of "X" broth were added to the sedimented cells, the suspension was heated at 85 to 90 C for 5 minutes, and the supernatant was collected after centrifugation. The supernatants were titrated for the V factor by diluting them in "X" broth and inoculating the dilutions with a drop of a twice-washed light suspension of *H. influenzae* in saline. The following controls with and without 0.1 per cent nicotinamide were also tested for the V factor: "X" broth, "XV" broth, "XV" broth heated a second time at 85 to 90 C for 5 minutes, and "X" broth to which 10 per cent erythrocytes had been added. The inoculated dilutions were examined for growth after 24 hours of incubation.

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Examination of table 1 reveals the destruction of the extracellular V factor on the addition of erythrocytes to chocolate broth and the prevention of this destruction by the addition of 0.1 per cent nicotinamide. Nicotinamide by itself or after incubation for 4 hours in the presence of 10 per cent erythrocytes did not act as V factor. There was no diminution in the intracellular V factor during the 4-hour incubation period.

Attempts to make use of the V-sparing property of nicotinamide by incorporating nicotinamide in blood agar were not successful. It was found that with longer incubation there was definite destruction of the V factor in the presence of nicotinamide. Furthermore, occasional strains of *Neisseria gonorrhoeae* were encountered which were inhibited by nicotinamide. The inability of nicotinamide to exert continued V-sparing activity and its effect on two strains of *N. gonorrhoeae* are demonstrated in the following experiment:

Blood agar (5 per cent human blood) with and without 0.1 per cent nicotinamide was compared with combination blood and chocolate agar (prepared by adding 5 per cent blood to cleared chocolate agar) containing different amounts of nicotinamide. From 30 to 60 minutes after preparation of the plates, each medium was streaked with light saline suspensions of two strains of *H. influenzae* and incubated in a candle jar at 37 C. A set of uninoculated plates was placed in the incubator at this time, and two sets were refrigerated at 7 C. Twenty-four hours later, the uninoculated incubated set and one of the refrigerated sets were streaked in the same manner with two strains of *H. influenzae* and two strains of *N. gonorrhoeae.* The remaining uninoculated set was streaked with the two strains of *H. influenzae* after 72 hours of refrigeration. All plates were examined for growth of the test organisms after 24 hours of incubation in the candle jar.

It was found that, at 37 C, the V factor supplied by the chocolate blood was destroyed by the unheated blood, even in the presence of 0.1 per cent nicotinamide. The combination of blood and chocolate agar is no better than the ordinary blood agar medium for the support of growth of *H. influenzae.* The presence of a small amount of nicotinamide in the medium, however, is of definite value since it permits the utilization of a small amount of the V factor of the unheated blood. The inhibition of a strain of *N. gonorrhoeae* (no. 593) as compared to another strain (no. 1007) was demonstrated. Further study showed that the maximum amount of nicotinamide that would not inhibit the growth of *N. gonorrhoeae* was approximately 0.005 per cent—an amount which produced only slight improvement in the growth of *H. influenzae.*

TABLE 1

The V-sparing activity of nicotinamide in chocolate broth

	DILUTIONS OF SUPERNATANTS							
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640
"XV" + 10% er. Sup.	0	0	0	0	0	0	0	0
"XV" + 10% er. Sup. H.	0	0	0	0	0	0	0	0
"XV" + 10% er. Sup. HC.	2	2	2	2	2	2	1	0
"XV" + 10% er. + nic. Sup.	2	2	2	2	2	1	0	0
"XV" + 10% er. + nic. Sup. H.	2	2	2	2	1	0	0	0
"XV" + 10% er. + nic. Sup. HC.	2	2	2	2	2	1	1	0
"X" + 10% er. Sup.	0	0	0	0	0	0	0	0
"X" + 10% er. Sup. H.	0	0	0	0	0	0	0	0
"X" + 10% er. Sup. HC.	2	2	2	2	2	2	1	0
"X" + 10% er. + nic. Sup.	0	0	0	0	0	0	0	0
"X" + 10% er. + nic. Sup. H.	0	0	0	0	0	0	0	0
"X" + 10% er. + nic. Sup. HC.	2	2	2	2	2	2	1	0
"X"	0							
"X" + nic.	0							
"XV"	2	2	2	1	1	0	0	0
"XV" H.	2	2	2	1	0	0	0	0
"XV" + nic.	2	2	2	1	1	1	±	0
"XV" + nic. H.	2	2	2	2	1	0	0	0

"X" = broth containing X factor; "XV" = cleared chocolate broth containing X and V factors; er. = erythrocytes; nic. = nicotinamide; Sup. = unheated supernatant; H. = heated; Sup. HC. = supernatant from heated cells; 2 = moderate growth; 1 = trace of growth; 0 = no growth.

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SUMMARY

The V factor of heated erythrocytes or of Lwoff and Lwoff yeast extract is destroyed by a heat-labile system of erythrocytes. This destructive action, like that of Mann and Quastel's "coenzyme nucleotidase," is retarded by nicotinamide. On continued exposure to a temperature of 37 C, however, marked destruction of the V factor occurs in the presence of as much as 0.1 per cent nicotinamide. In concentrations greater than 0.005 per cent, nicotinamide inhibits the growth of some strains of *Neisseria gonorrhoeae*.

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PENICILLIN PRODUCTION BY A THERMOPHILIC FUNGUS

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A program for obtaining antibiotics from thermophilic organisms active against *Brucella abortus* is under way in this laboratory. Several fungi, actinomycetes, and bacteria, all growing well at 52 C and forming substances inhibitory to *Brucella* as well as to many other common test bacteria, have been isolated in pure culture from composted manure. One of the fungus cultures is unique in that it produces penicillin at 52 C and is a new species member of the group of penicillin-producing fungi. Hitherto, the highest temperature for penicillin formation has been 37 C (Foster and Karow, 1945). The organism has been identified tentatively by Dr. C. W. Emmons, Principal Mycologist of the National Institute of Health, as *Malbranchea pulchella.*

In liquid media, it makes very slow and poor growth at room temperature; at 37 C the growth is substantially less than it is at 52 C, at which temperature growth is very rapid and abundant in the common culture media and is especially luxuriant in a corn steep liquor medium. In surface culture, maximum activity is reached about the thirteenth day at 37 C and about the fourth or fifth day at 52 C. In shake cultures the maximum is reached in 4 days. Maximum broth potency is of the order of approximately 5 international units per ml. Consequently, for routine study the cup assay procedure was abandoned in favor of the serial dilution method. The low activity doubtless is due partially to the rapid decomposition rate of penicillin at this elevated temperature. Potencies generally range between 1:64 to 1:128 against *Staphylococcus aureus* H.

Of several media tested, corn steep liquor medium gives the highest antibacterial activity. The active substance was identified as penicillin by the following criteria:

(1) The temperature stability corresponds to that of authentic penicillin compared simultaneously.

(2) The pH stability corresponds to that of authentic penicillin compared simultaneously.

(3) Solubility characteristics: fat solvents including amyl acetate do not extract the active factor at neutral pH, but do at pH 2 to 4.

(4) The antibacterial activity of broths and solvent-prepared concentrates is destroyed by penicillinase (clarase) as is authentic penicillin simultaneously.

(5) The antibacterial spectrum of a concentrate against 17 different bacteria matched closely that of authentic penicillin run simultaneously. The minor differences observed are what one might expect between different penicillins.

(6) As with all penicillin-producing fungi, maximum activity is produced in corn steep liquor medium.

(7) The addition of penicillin precursors, phenylacetic acid or phenylacetamide, stimulates the production of antibacterial activity over controls without this supplement, a behavior characteristic of other penicillin-producing fungi. In one case a 4-fold stimulation was obtained.

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STUDIES ON STREPTOMYCIN

I. Factors Influencing the Activity of Streptomycin

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The interpretation of studies on an antibacterial substance *in vitro* and an understanding of its activity *in vivo* depend upon certain variables known to be operative in either or both conditions. Appreciation of such variables permits a more logical approach to the chemotherapeutic use of a drug and also may explain certain discrepancies seen not only between results *in vitro* and *in vivo*, but also between studies *in vitro* conducted by different groups of investigators. There is adequate indication already that the activity of streptomycin is influenced considerably by certain variations in its environment. Since streptomycin shows definite promise as a chemotherapeutic substance, it was deemed advisable to study some of these factors in detail.

The effect of salts. Foster and Woodruff (1943) found that certain salts suppress the activity of streptothricin against *Escherichia coli* in nutrient broth. Loo *et al.* (1945) reported that the addition of phosphate and other salts to streptomycin solutions caused a marked increase in the size of the zone of inhibition in the paper-disc plate method of assay. On the other hand, it has been reported recently (Klein and Kimmelman, 1946) that 0.5 per cent sodium chloride shows some antagonistic effect toward streptomycin activity on shigellae.

In our studies of the factors affecting the activity of streptomycin[2] it was observed that salts markedly decrease its activity. The salts used were sodium chloride, potassium chloride, sodium sulfate, sodium tartrate, Soerensen's buffer, and ammonium acetate in concentrations of 0.5, 0.85, and 2.0 per cent. The salt solutions were adjusted to the pH of Difco nutrient broth (pH 6.8) and added in the ratio of 0.1 ml of the salt solution to 0.9 ml of the medium. The salts and streptomycin were added individually to the medium immediately prior to the addition of the organisms.

The organisms used in this set of experiments included two strains of *Staphylococcus aureus*, two strains of *Proteus vulgaris*, one strain of *Shigella dysenteriae* (Shiga), one strain of *Eberthella typhosa*, two strains of *Bacillus cereus*, one strain of *Bacillus anthracis*, and one strain of *Bacillus subtilis*.

Table 1 shows the results of an experiment using sodium chloride in concentrations of 0.5, 0.85, and 2.0 per cent. These findings are typical of those obtained from 20 experiments. It is apparent that the salt greatly reduced the ability of streptomycin to inhibit growth of all the strains tested. In each case, the amount of streptomycin rendered ineffective was dependent upon the concentration of salt present. Essentially the same results were obtained consistently with the other five salts, differences being only quantitative.

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[1] With the technical assistance of Renate Hirsch and Olga Majocchi.

[2] Six samples of streptomycin were used in these studies. They included (1) streptomycin sulfate commercial, Pfizer, lots 455 and 456, (2) streptomycin sulfate commercial, Abbott, lot G602 A600, (3) streptomycin hydrochloride commercial, Merck, lot 226, (4) streptomycin sulfate, Parke, Davis, 800 μg per mg, (5) streptomycin sulfate, Abbott, 700 μg per mg, and (6) streptomycin sulfate, Pfizer, 830 μg per mg. Similar results were obtained with all the samples.

Since salts had such a marked effect in reducing the activity of streptomycin, studies were made of their effect on the assay of streptomycin, as proposed by Loo *et al.* (1945), in which *B. subtilis* 04528 is the test organism. Sodium chloride and sodium sulfate were added individually to the medium in concentrations of 0.85 and 2.0 per cent, and then solutions of streptomycin were assayed in the usual manner. Two per cent of either of these salts in the medium completely neutralized the activity of streptomycin, as indicated by the absence of zones of inhibition. When the medium contained 0.85 per cent salt, zones of inhibition appeared only with the highest concentration of streptomycin tested (32 µg per ml). These zones were only 16 and 15 mm in diameter with the two salts, respectively, as compared to the control of 26 mm.

TABLE 1

The effect of sodium chloride on the activity of streptomycin

NaCl CONCENTRATION..........	0	0.5%							0.85%										2%								
Streptomycin µg/ml	MIC*	0.5	2	5	10	25	50	75	0.5	2	5	10	25	50	75	100	200	400	0.5	2	5	10	25	50	100	200	400
Organism																											
S. aureus I..................	2.0	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	−	−
S. aureus II..................	1.0	+	+	+	+	−	−	−	+	+	+	+	+	−	−	−	−	−	+	+	+	+	+	+	+	−	−
E. typhosa..................	1.0	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	−	−
P. vulgaris S..................	2.0	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	−
P. vulgaris R..................	8.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
B. subtilis 04528..................	8.0	+	+	+	+	+	−	−	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+
B. anthracis..................	0.1	+	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−
S. dysenteriae 42..................	1.0	+	+	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−
B. cereus I..................	1.0	+	+	+	−	−	−	−	+	+	+	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−
B. cereus II..................	1.0	+	+	+	−	−	−	−	+	+	+	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−

The tubes were incubated at 37 C.

+ = visible growth by 4 days.

The medium used in these experiments was Difco nutrient broth.

The inoculum for each tube was 0.05 ml of a faintly turbid water suspension of the organism. Final volume in each tube was 1.0 ml.

*MIC = minimal inhibiting concentration (no visible growth in 4 days).

Loo *et al.* reported that the presence of salts in solutions to be assayed, except when in high concentrations, did not affect the results of assay if the streptomycin samples were prepared in 0.1 M phosphate buffer, pH 7.8. They reported an increase in the size of the zone when the solution to be assayed contained high salt concentrations. In our experience the inclusion of 2.0 per cent sodium chloride, potassium chloride, or ammonium acetate and 0.85 per cent sodium chloride or ammonium acetate to the streptomycin sulfate solutions prepared for assay in 0.1 M phosphate buffer resulted in slightly decreased zones of inhibition, especially in the lower concentrations of streptomycin.

The zone diameters obtained from streptomycin solutions containing 2.0 per cent ammonium acetate were from 1 to 2 mm smaller than those obtained in the absence of the salt.

To determine whether the salts contained in blood and urine would interfere with the assay of streptomycin in these body fluids, streptomycin in concentrations of 10, 25, 50, 100, and 500 μg per ml was added to defibrinated sheep blood and normal human urine.[3] There was no apparent loss in activity after an interval of 3 hours at room temperature or 4 C, as measured by the disc assay. In another experiment streptomycin was added to the same body fluids in concentrations of 50 and 100 μg per ml and held at 37 and 4 C overnight and then assayed. Again there was no demonstrable loss in activity. The cause for the absence of the salt effect in these body fluids is not apparent. It is possible, however, that some substance is blocking the salt effect in these instances.

The antagonism of streptomycin by salts is not due to stimulation of the growth of the test organisms by the salts since no difference in growth rate was observed in controls run with and without salts in the absence of streptomycin. In these experiments growth was followed turbidimetrically in the Klett-Summerson photoelectric colorimeter in the presence and absence of potassium chloride, sodium chloride, and ammonium acetate in concentrations of 0.5, 0.85, and 2.0 per cent.

There are at least two possible explanations for the salt antagonism. There may be a direct action by the salt on streptomycin, producing a complex, or the origin of this phenomenon may be the result of an interaction with the bacteria.

An attempt was made to determine whether the effect was a direct one on streptomycin. Solutions of streptomycin (15,000 μg per ml) were prepared in 5.0 per cent ammonium acetate and 5.0 per cent sodium chloride. Aliquots of these solutions were held at room temperature and 4 C and assayed at intervals of 4, 10, and 16 days. The samples for assay were diluted until the salt concentration was below that which affected the activity of streptomycin. No decrease in the activity of streptomycin was observed. The inadequacy of this approach is obvious. In the first place, any direct effect by salt on streptomycin may well be reversible. Secondly, such a direct effect may follow the law of mass action, so that for a particular degree of effect a constant ratio of salt to streptomycin must exist.

The effect of inoculum size. The proliferation of four different-sized inocula of *S. aureus*, *P. vulgaris*, *E. coli*, and *S. dysenteriae* (Sonne) in varying concentrations of streptomycin was determined turbidimetrically.[4] The results of a typical experiment with *S. aureus* are shown graphically in figure 1. This type of experiment was repeated several times with *S. aureus* and the other organisms with essentially the same results (in the case of the other bacteria slightly different ranges of concentrations of streptomycin were required).

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[3] One and one-half per cent ash was recovered from the urine.

[4] Measurements were made with the Klett-Summerson photoelectric colorimeter using the number 42 blue filter. There was a linear relationship between the number of bacteria in suspension and the Klett readings.

Without exception, the larger the initial inoculum, the sooner multiplication began in any particular concentration of streptomycin. The number of viable organisms originally present is a very critical factor insofar as multiplication in any one concentration of streptomycin is concerned.

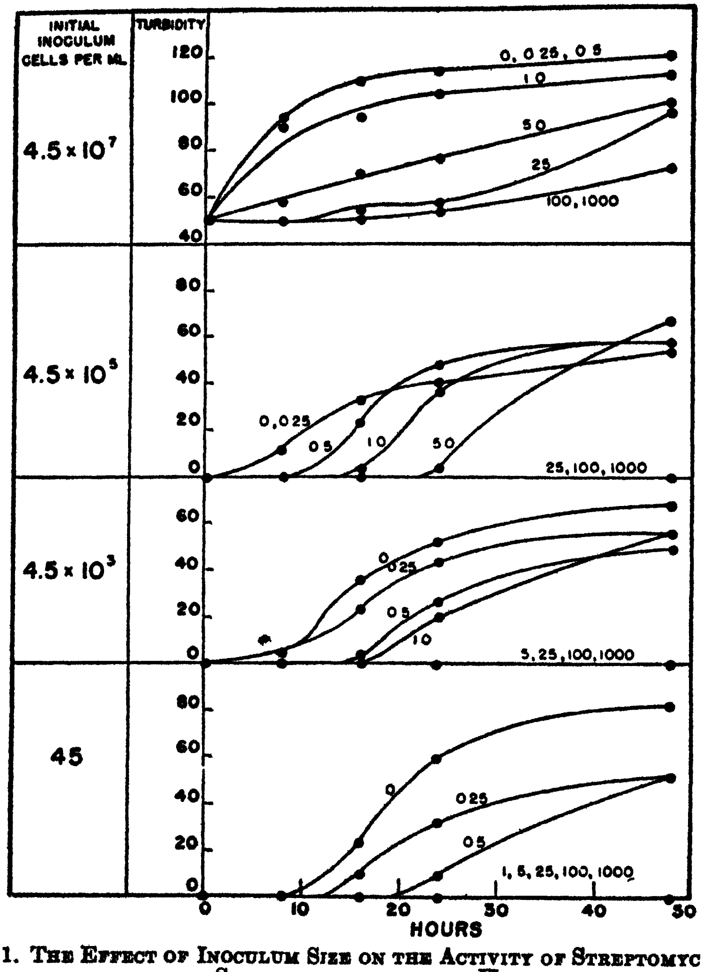

FIG. 1. THE EFFECT OF INOCULUM SIZE ON THE ACTIVITY OF STREPTOMYCIN ON STAPHYLOCOCCUS AUREUS II

Figures adjacent to plotted curves represent streptomycin concentration in μg per ml.

If the assumption is made that all cells in a given sample are relatively homogeneous with respect to susceptibility to an inhibitor of cell division, then the degree of inhibition produced by a certain concentration of that inhibitor should be the same whether one cell or one billion cells are present (Henry and Henry, 1945). If this does not hold in a particular case, as in the case of streptomycin, there are at least four possible explanations:

(1) Sufficient inhibitor might be removed from the medium by adsorption onto cellular substances to result in considerably reduced concentrations. The amount adsorbed would be directly proportional to the number of cells present. In many instances multiplication became apparent only after 2 or 3 days' incubation. This observation does not favor such an explanation, since adsorptions are not usually that slow. The supernatants of cultures grown for 3 days in the presence of various concentrations of streptomycin were assayed for streptomycin. There was no significant decrease in the concentration of streptomycin ascribable to adsorption onto the cells.[5] This hypothesis, therefore, can be rejected.

(2) The inhibitor may be destroyed by the bacteria or by the temperature of incubation. This also may be rejected by the findings given above under (1).

(3) There may be a streptomycin antagonist produced by the bacteria; the quantity of antagonist produced would parallel the number of cells present. To test this possibility, supernatants similar to those examined in (1) above were reinoculated with fresh inocula containing varying numbers of cells. The results were qualitatively identical to those seen in figure 1; again there was in the case of rather small inocula, the same lag period of 24 to 48 hours before multiplication progressed to the point at which there was detectable turbidity. This would appear to eliminate such a hypothesis for the phenomenon observed.

(4) The assumption that the population is relatively homogeneous with respect to susceptibility to the inhibitor is false. The population is very heterogeneous in that there is a wide range of sensitivity of individual bacteria. At a certain concentration of streptomycin there may be very few or no bacteria present in the initial inoculum sensitive to that concentration. In such a case the quantitative increase in number of cells with time does not differ detectably from that of a similar sample without streptomycin. At a somewhat higher concentration of streptomycin, many cells are either partially or completely inhibited, and although they contribute to the original turbidity and presumably to future measurements made (though they may be dead in the latter case), they do not divide and thus give no increase in turbidity. Rather, it is those cells which are resistant to that concentration of streptomycin which multiply. As the concentration of streptomycin is increased, the relative number of cells resistant to that particular concentration decreases. Since there are fewer cells able to multiply, a longer period of time would elapse before there would be an increase in measurable turbidity. Eventually a concentration is reached to which no cells present are inherently resistant.

The larger the initial inoculum, the greater is the actual number (not relative number) of organisms resistant to any one concentration. Therefore, a detectable increase in turbidity at that concentration would appear sooner. Conversely, as the initial inoculum is decreased, longer periods of incubation are required for increased turbidity because of the fewer organisms initially present which are resistant to that concentration of streptomycin. Eventually an inoculum size is reached at which the statistical probability of having any organisms present at all which are resistant to that concentration is very small. In such a case, multiplication would occur only rarely.

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[5] Actually, in certain media a significant drop in activity of streptomycin occurred. A similar decrease, however, was found in sterile controls. This drop was not due, therefore, to removal of the streptomycin from solution by the bacteria. This phenomenon is being studied in greater detail and will be reported in a subsequent paper of this series.

This thesis is compatible in every way with the results observed with streptomycin. According to this hypothesis, the bacteria growing out of any one concentration of streptomycin should be resistant to that concentration. Such cells were removed by centrifugation from the media in which they had been grown and resuspended in fresh media at the same concentration of streptomycin. It was found that there was no delay in growth, that the turbidity increased as did the controls without streptomycin. These same cells multiplied equally well in concentrations double that in which they grew originally. Since the susceptible cells had been eliminated from the strain by exposure to streptomycin, the cells resistant to high concentrations of streptomycin would presumably be present in a much higher ratio than originally.

Such a "weeding out" process may be the explanation for the phenomenon of "development of resistance" to streptomycin *in vitro* and *in vivo* (Finland *et al.*, 1946; Miller and Bohnhoff, 1946; Wolinsky and Steenken, 1946; Youmans and Hinshaw, 1946). In fact, the recent studies reported by Klein and Kimmelman (1946) indicate that this is the principal, if not the only, factor in this phenomenon. It must be emphasized, however, that the results presented here do not necessarily exclude the possibility that any one bacterium can become increasingly resistant to streptomycin as a response to the stimulus offered by the streptomycin.

The effect of nutritional environment and serum proteins. The type of experiment discussed in the previous section was conducted with *S. aureus* in three media, a relatively poor medium for growth (a semisynthetic medium) and two enriched media.[6] Similar results were obtained with the three media except that in the richer media the organisms growing out of a particular concentration of streptomycin multiplied at a faster rate, and thus increases in turbidity usually appeared sooner than in the case of the poorer medium. In no case, however, did organisms grow in higher concentrations of streptomycin in the richer media than in the poorer medium. It is doubtful whether slight differences of streptomycin activity in these various media would have been detected in these experiments since the concentrations of streptomycin used ranged in steps of from 2- to 10-fold. Wallace *et al.* (1945), working with *E. typhosa* and *S. aureus* in nutrient broth and a brain heart infusion broth low in salt content, obtained results which they interpreted as indicating that the latter medium contained an antagonist for streptomycin. Our results are compatible with these, but the point of difference is in the interpretation.

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[6] The semisynthetic medium was that of Landy and Dicken (1942) with the omission of sodium acetate, asparagine, guanine, xanthine, and uracil. One of the richer media consisted of beef extract, 0.5 per cent; yeast extract, 0.3 per cent; proteose-peptone no. 3, 0.5 per cent; and lactose, 0.5 per cent. The other was Difco nutrient broth to which was added yeast extract, 0.3 per cent, and glucose, 0.5 per cent.

The activity of streptomycin against the *E. coli* and *S. aureus* strains was studied in the presence and absence of horse serum (1 and 5 per cent). Within the limitations of the turbidimetric method used, there was no indication of antagonism of activity. This is in confirmation of previous reports (Price *et al.*, 1946; Elias and Durso, 1945; Reimann *et al.*, 1945).

The effect of pH. In confirmation of other reports (Waksman and Schatz, 1945; Wolinsky and Steenken, 1946), the activity of streptomycin was found to decrease with decreasing pH. The effect of low pH on streptomycin activity (pH 3.0 for 24 hours) is reversible, as indicated by the fact that such solutions of streptomycin regained their original activity at pH 7.8. This is in agreement with the findings of Wolinsky and Steenken (1946).

Indication of an antagonist for streptomycin. Filtrates of a strain of *S. aureus* and one of *P. vulgaris* grown in the medium suggested by Waksman and Schatz (1945) for 5 days at 37 C antagonized the activity of streptomycin against 3 strains of *S. aureus* grown in the same medium. The filtrate reversed the activity of 50 μg per ml of streptomycin at pH 6.0 in a dilution of 1:50. The same concentration of streptomycin inhibited growth in the controls. Since this factor was active only in acid pH, in which streptomycin is relatively ineffective, it appears to be of little physiological importance.

DISCUSSION

It is apparent that reports concerning the sensitivity of a given strain of bacteria to streptomycin *in vitro* are valid only for the specific conditions under which the particular test was conducted. The activity of streptomycin as judged from bacterial multiplication is affected to a very marked degree by variables such as salt concentration, inoculum size, nutritional environment, pH, and time at which the sensitivity test is read. Since the conditions for such tests have not been standardized, it would be impossible to compare the results reported in the literature on the susceptibility *in vitro* of various bacteria. Thus in our preliminary work with the *S. aureus* strain, it was found that, based on visible growth at 24 hours, the bacteria were resistant to 10 μg per ml and susceptible to 25 μg per ml. As seen in figure 1, however, the great majority of bacteria in this strain are completely inhibited at 0.5 μg per ml. It is, therefore, suggested that for such *in vitro* sensitivity tests, some standard conditions of these variables should be adopted universally so that the results would be suitable for comparison.

One of the uses of such data obtained *in vitro* is the indication given as to whether an infection by the strain in question would be amenable to therapy, and also as to the blood level required for effective therapy. Thus, for sensitivity tests it would seem that conditions should simulate the *in vivo* environment insofar as possible.

The fact that in any strain of bacteria there are some organisms very resistant to streptomycin would indicate the advisability of using the highest dosages within clinically safe limits when treating an infection with streptomycin.

SUMMARY

Sodium chloride, potassium chloride, sodium sulfate, sodium tartrate, Soerensen's buffer, and ammonium acetate in physiological concentrations antagonized the activity of streptomycin against *Staphylococcus aureus*, *Proteus vulgaris*, *Shigella dysenteriae* (Shiga), *Eberthella typhosa*, *Bacillus cereus*, *Bacillus anthracis*, and *Bacillus subtilis*. The degree of antagonism was directly proportional to the salt concentration.

The activity of streptomycin varied inversely with the size of initial inoculum. This effect is apparent rather than real and arises from the fact that, the larger the inoculum, the greater the number of resistant organisms present.

Evidence is presented for the presence of a weak antagonist of streptomycin in the filtrates of cultures of *S. aureus* and *P. vulgaris*. This factor appears to be of little physiological importance since it was active only in acid pH.

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THE EFFECT OF STREPTOCOCCUS PYOGENES EXTRACTS AND FILTRATES ON VARIOUS BACTERIA

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Bacteria as a source of antibiotic materials have been the object of intensive study. Some of the bacteria more recently investigated for antibiotic properties have been *Chromobacterium violaceum* by Lichstein and Van de Sand (1945) and the *Bacillus subtilis* group by various workers, among whom may be mentioned Salle and Jann (1945), Johnson, Anker, and Meleney (1945), Olivier, De Saint Rat, and Corvasier (1945), and Olivier (1946). Waksman (1941) and Waksman and Woodruff (1942) reviewed the earlier work in which bacteria were used as the source of antibiotic substances. Since most of the antagonistic bacteria thus far investigated are rod forms common to soil, air, and water, it was felt that the reactions of pathogenic gram-positive cocci would be interesting. Accordingly, investigations of the effects of extracts of *Staphylococcus aureus* organisms and of the media in which they were grown were made and the results reported in a previous paper (Nutini, Kelly, and McDowell, 1946). On the basis of earlier work with protein-free extracts of animal tissues (Nutini and Kreke, 1942; Nutini and Lynch, 1945; Nutini, Kreke, and Schroeder, 1945; Nutini, Thomas, and Smolar, 1945), the extracts of the organism were prepared in the same manner. In conjunction with this study, the effects of ultraviolet irradiation on the organisms and their media were observed. The present work is a continuation of this study using *Streptococcus pyogenes* as a source of material. The preparations consisted of (1) a protein-free alcohol extract of *Streptococcus pyogenes* cells; (2) a protein-free alcohol extract of the medium in which ultraviolet-irradiated and nonirradiated cultures of *Streptococcus pyogenes* were grown, and (3) an untreated sterile filtrate of the media from irradiated and nonirradiated cultures of *Streptococcus pyogenes*.

METHODS

The culture of *Streptococcus pyogenes* no. 6636 was secured from the American Type Culture Collection.

Cell extract. The cell extract was prepared as described in a previous paper (Nutini, Kelly, and McDowell, 1946) except that Difco brain-heart infusion made up with 1.5 per cent agar was used instead of nutrient agar as the medium in the Roux flasks. The inocula were from 24-hour cultures of *Streptococcus pyogenes* in brain-heart infusion broth.

Extracts and filtrates of medium from nonirradiated cultures. With the exception that brain-heart infusion broth was used instead of nutrient broth as the culture medium, the extracts and filtrates from nonirradiated cultures were prepared in essentially the same manner as that described by Nutini, Kelly, and McDowell (1946).

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Extracts and filtrates from irradiated cultures. In this part of the work the method described in the previous paper was modified somewhat. Approximately 50 ml of brain-heart infusion broth freshly inoculated with a culture of *Streptococcus pyogenes* was poured aseptically into sterile special glass tubes (Corning 9741, grade 3) and incubated at 37 C for 48 hours at a distance of about 45 cm from a Sperti "mercolite" lamp. The ultraviolet irradiation was from above and the tubes were rotated from time to time. At the end of 48 hours extracts and filtrates were prepared as described in the previous paper. The broth in the tubes was turbid, but the growth was not so heavy as that in nonirradiated tubes.

Measurement of activity. Bacterial growth of the test organisms was measured in terms of increase or decrease in the number of colonies by the pour plate method exactly as described for the work with *Staphylococcus aureus* with the following exceptions: Five-tenths ml of the test organisms were used instead of 0.1 ml. *Streptococcus pyogenes* was used in dilutions of 1:10 or 1:100, rather than 1:10,000, and was streaked uniformly over the surface of prepared brain-heart agar plates. The organisms used to test the effect of extracts of the *Streptococcus pyogenes* cells were *Staphylococcus aureus*, *Escherichia coli*, *Aerobacter aerogenes*, *Shigella dysenteriae*, *Streptococcus pyogenes*, *Corynebacterium diphtheriae*, *Eberthella typhosa*, *Salmonella enteritidis*, *Salmonella paratyphi*, *Bacillus subtilis*, and *Diplococcus pneumoniae*, type I. Only the first 5 were used for the extracts and filtrates of the media. Control plates containing the test organisms and agar (nutrient or brain-heart, depending on the organism tested) or agar and sterile brain-heart infusion broth were included in the series. All tests were made in triplicate.

The percentage of inhibition or stimulation of growth of the test organisms was calculated with the control plates serving as the 100 per cent point of reference. Results lying in the range of 25 per cent stimulation or inhibition were listed as representing no effect in order to be certain that the results were beyond the limits of experimental error.

Diplococcus pneumoniae was the only test organism to be effectively inhibited by the *Streptococcus pyogenes* cell extract, and because of this biochemical tests including the fermentation of lactose, sucrose, and glucose, the coagulation of litmus milk, the liquefaction of gelatin, and bile solubility were made in triplicate and limited to this organism.

RESULTS

The various reactions of the 11 test organisms to extracts of cells of *Streptococcus pyogenes*, and to extracts and filtrates of the media in which they were grown, are given in tables 1 and 2. Growth varied with the species of the organism tested, with the extract or filtrate used, and in many cases with the concentration of the testing material. There was no consistent difference in the results obtained with gram-positive and gram-negative organisms or with filtrates and extracts of broth media from irradiated organisms.

Broth extracts. The extracts of broth media both from irradiated and nonirradiated *Streptococcus pyogenes* produced predominantly inhibitory effects on the growth of test organisms with the exception of that of *Streptococcus pyogenes, Staphylococcus aureus,* and *Shigella dysenteriae.*

Filtrates. Either inhibitory or stimulatory effects were produced by the filtrates of the broth medium from irradiated and nonirradiated cultures of *Streptococcus pyogenes,* depending on the organism and the concentration of the filtrate used. *Streptococcus pyogenes* was stimulated.

TABLE 1

Effect on the growth of bacteria of an extract of the cells and of filtrates and extracts of irradiated and nonirradiated broth cultures of Streptococcus pyogenes

TYPE OF EXTRACT USED	%	TEST ORGANISMS				
		E. coli	*A. aerogenes*	*S. dysenteriae*	*S. aureus*	*S. pyogenes*
Cell extract	0.1	No effect	Slight inh.	Slight inh.	Slight inh.	Stimulation
	0.5	Slight inh.	Slight inh.	No effect	Slight inh.	Stimulation
	1.0	Slight inh.	No effect	No effect	Stimulation	Stimulation
	5.0	No effect	No effect	No effect	Stimulation	Stimulation
Extract of broth media from nonirradiated *S. pyogenes*	0.1	Slight inh.	No effect	No effect	Stimulation	Stimulation
	0.5	Slight inh.	Slight inh.	No effect	No effect	Stimulation
	1.0	Slight inh.	Slight inh.	Stimulation	No effect	Stimulation
	5.0	Slight inh.	Slight inh.	No effect	Slight inh.	Stimulation
Extract of broth media from 48-hr irradiated *S. pyogenes*	0.1	No effect	Slight inh.	Stimulation	Slight inh.	Inhibition
	0.5	No effect	Inhibition	Slight inh.	Slight inh.	Stimulation
	1.0	No effect	Inhibition	Slight inh.	Slight inh.	Stimulation
	5.0	Complete inh.	Inhibition	Complete inh.	Inhibition	Complete inh.
Filtrate of broth media from nonirradiated *S. pyogenes*	0.1	No effect	Slight stim.	No effect	Slight inh.	Slight stim.
	0.5	No effect	No effect	Slight inh.	Slight inh.	Stimulation
	1.0	No effect	Stimulation	Slight inh.	Slight inh.	Stimulation
	5.0	Slight inh.	Stimulation	Inhibition	Slight inh.	Stimulation
Filtrate of broth media from 48-hr irradiated *S. pyogenes*	0.1	No effect	Slight inh.	No effect	Slight stim.	Slight stim.
	0.5	No effect	No effect	Slight inh.	Slight stim.	Slight stim.
	1.0	Slight stim.	Slight inh.	Slight inh.	No effect	Stimulation
	5.0	Slight stim.	Slight inh.	Slight inh.	No effect	Stimulation

Control colonies, 100 per cent; slight stimulation, 125 to 175 per cent; slight inhibition, 75 to 25 per cent; inhibition, 25 to 0 per cent; stimulation, 175 to 500 per cent; no effect, 75 to 125 per cent.

Cell extract. Extracts of *Streptococcus pyogenes* cells had for the most part an inhibitory or little effect on the growth of most of the bacteria tested. *Streptococcus pyogenes,* however, was stimulated at all concentrations, and *Staphylococcus aureus* was stimulated at the two highest concentrations.

The effects of cell extracts of *Streptococcus pyogenes* on additional bacteria are given in table 2. The organisms listed in table 1 are also included for purposes of comparison. The data in table 2 show that in general the gram-negative organisms are inhibited, the gram-positive ones stimulated, by extracts of *Streptococcus pyogenes* cells.

Biochemical changes. In concentrations of 5 per cent the cellular extract of *Streptococcus pyogenes* inhibited the fermentation of sucrose, lactose, and glucose by *Diplococcus pneumoniae,* type I. The coagulase system as determined by the coagulation of litmus milk was affected in all but the 5 per cent concentration of the cellular extract when *Diplococcus pneumoniae* was used as the test organism.

DISCUSSION

Although emphasis has been placed upon the inhibitory effect of many bacteria on the growth processes of other bacteria, the literature as well as the experiments here presented give ample evidence of both stimulatory and inhibitory effects produced by the extracts of the same bacteria and of the media in which they were grown. Green (1940) attributed the stimulatory effects from *Brucella abortus* extracts to a substance which he called the "P" or proliferation factor.

TABLE 2

Effect of an alcoholic extract of cells of Streptococcus pyogenes on the growth of certain bacteria

ORGANISM TESTED	PERCENTAGE OF EXTRACT USED			
	0.1	0.5	1.0	5.0
Gram-negative organisms				
S. paratyphi	No effect	No effect	No effect	Slight inh.
E. typhosa	Slight stim.	Slight stim.	Slight stim.	No effect
S. enteritidis	Slight inh.	Slight inh.	Slight inh.	Inhibition
E. coli	No effect	Slight inh.	Slight inh.	No effect
A. aerogenes	Slight inh.	Slight inh.	No effect	No effect
S. dysenteriae	Slight inh.	No effect	No effect	No effect
Gram-positive organisms				
S. aureus	Slight inh.	Slight inh.	Stimulation	Stimulation
S. pyogenes	Stimulation	Stimulation	Stimulation	Stimulation
C. diphtheriae	Slight stim.	Stimulation	Stimulation	Stimulation
B. subtilis	Slight stim.	No effect	No effect	Slight inh.
D. pneumoniae, type I	Inhibition	Inhibition	Inhibition	Inhibition

Control colonies, 100 per cent; slight stimulation, 125 to 175 per cent; slight inhibition, 75 to 25 per cent; inhibition 25 to 0 per cent; stimulation, 175 to 500 per cent; no effect, 75 to 125 per cent.

In the present study the reaction of *Streptococcus pyogenes* to the various extracts of *Streptococcus pyogenes* cells and extracts and filtrates of the media in which they were grown was more constant than was the case with other test organisms. There are many reports of investigations on the effect of extracts of streptococcal cells on streptococci (Lewandowski, 1944; Fleming, 1940; Blundell, 1942; Meyer, Hobby, Chaffee, and Dawson, 1940), but studies of the action of this organism and its products on organisms other than the streptococci are few. Le Chuiton, Bideau, Pennaneac'h, and Mollaret (1938) found that one strain of streptococci was inhibitory to the growth of *Corynebacterium diphtheriae* whereas the results here presented show a decided stimulation of *Corynebacterium diphtheriae* by the cellular extract. This difference may well be due to difference in the streptococcal preparation and in the strains of organisms used.

The probable mechanisms involved have been discussed elsewhere (Waksman, 1944), and it is felt that further investigations are necessary before definite statements can be made regarding the mode of action of these extracts and filtrates.

SUMMARY

Protein-free alcoholic extracts and untreated filtrates of the media in which *Streptococcus pyogenes* was grown were prepared and tested for effects on the growth of *Staphylococcus aureus*, *Escherichia coli*, *Shigella dysenteriae*, *Aerobacter aerogenes*, and *Streptococcus pyogenes*.

The alcoholic extracts of *Streptococcus pyogenes* cells in various concentrations were tested for effect on the growth of the foregoing bacteria as well as on *Diplococcus pneumoniae*, *Salmonella paratyphi*, *Salmonella enteritidis*, *Eberthella typhosa*, *Bacillus subtilis*, and *Corynebacterium diphtheriae*.

In general, the growth of gram-negative organisms, except *Eberthella typhosa*, was inhibited by the alcoholic cellular extract, and all of the gram-positive bacteria with the exception of *Diplococcus pneumoniae*, type I, were stimulated by this extract.

The extracts and filtrates of the media in which *Streptococcus pyogenes* was grown produced predominantly inhibitory effects. *Streptococcus pyogenes*, however, was stimulated greatly by all the preparations except the extract of the broth media of the 48-hour ultraviolet-irradiated culture of *Streptococcus pyogenes*, which produced an inhibition of growth at the lowest and highest concentrations.

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THE RELATION OF PROTEIN BINDING TO THE PHARMACOLOGY AND ANTIBACTERIAL ACTIVITY OF PENICILLINS X, G, DIHYDRO F, AND K[1]

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It is well recognized that many substances which inhibit bacterial growth *in vitro* may lose a part or all of this antibacterial activity in the presence of human serum. This phenomenon is attributed in part to the fact that the antibacterial agents are adsorbed or otherwise bound to some constituent of serum. The role of the serum proteins in this process has recently been reviewed by Davis (1946). It has been demonstrated by Davis (1942, 1943) and by Gilligan (1943) that the individual sulfonamides are bound to serum albumin to a varying degree, and there is suggestive evidence that the portion of the drug which is bound is inactive against bacteria.

Chow and McKee (1945) have shown by dialysis experiments that crystalline penicillin (G) is bound to serum albumin. Similar results were reported from this laboratory in experiments in which whole serum and crude penicillin were used (McDermott and Nelson, 1945). In the latter experiments, the antibacterial activity of the penicillin-serum complex was not studied. Chow and McKee believed, however, that, although the penicillin was bound to a significant degree, the penicillin-albumin complex was still fully active. In support of this hypothesis is the fact that early studies of penicillin had indicated that human serum had no inhibitory effect upon the action of the drug (Abraham *et al.*, 1941; Rammelkamp and Keefer, 1943).

In contrast to these findings, it was noted by Romansky (1944) and by Bigger (1944) that the presence of serum may interfere with the bioassay of penicillin in the lowest range of the broth dilution methods. Bigger also reported that penicillin is inactivated by incubation in human serum at 37 C. Both of these observations have been repeatedly confirmed in this and in other laboratories, although opinions as to the nature of the phenomena have differed. Additional relevant observations are those of Holmes and Lockwood (1944), who noted the presence of an "antipenicillin factor" in human ascitic fluid and in the serum of humans, horses, and rabbits. The substance responsible for this antipenicillin effect was dialyzable and relatively heat-stable.

Thus, by the end of 1945, evidence had been accumulated which would indicate: (a) that penicillin G would bind to purified albumin and to a constituent of serum which presumably was albumin; and (b) that there was a substance or substances in human serum which could destroy or otherwise inactivate penicillin *in vitro*.

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[1] This investigation was conducted with a grant-in-aid from the National Institute of Health. The study was also aided in part by a grant from the Lederle Laboratories, Pearl River, New York.

Early in 1946 it was first observed that there is a marked discrepancy between the *in vitro* and the *in vivo* (mouse and rabbit) activity of the four penicillins X, G, F, and K (Coghill *et al.*, 1946; Eagle and Musselman, 1946; Hobby *et al.*, 1946). The disparity was striking with penicillin K, which is the most active of the four *in vitro* but is the least active in the treatment of streptococcus infections in mice. In the rabbit, penicillin K is notably less effective in the treatment of syphilis than are penicillins X or G. In man, comparable quantitative observations of the therapeutic effectiveness of penicillin K are not yet available. It was noted, however, in three different laboratories (Coghill *et al.*, 1946; Eagle and Musselman, 1946; McDermott and Tompsett, 1946), that penicillin K apparently disappears from the circulating blood of man with unusual rapidity following parenteral administration in doses of 20,000 to 90,000 units. As the urinary excretion of the penicillin K was notably low in these experiments, it was concluded that the material was destroyed within the body. It was assumed that the apparently rapid disappearance of the penicillin from the blood of man would be responsible for a relatively low therapeutic effectiveness similar to that observed in the mouse and the rabbit.

These two characteristics of penicillin K—a marked difference between its *in vitro* and *in vivo* activity, and a pharmacologic behavior apparently different from closely related compounds—suggested that the phenomenon of protein binding might be operative. Accordingly, an investigation has been conducted of the role of the serum proteins in the pharmacology of penicillin K and the other individual penicillins.

METHODS AND MATERIALS

The method of Hobby (1946) for the assay of penicillin in serum was modified for the purposes of this investigation. The procedure differs from the more commonly used methods in that the increments are smaller, the amount of serum is kept constant in each tube by the addition of normal, pooled human serum, and the inoculum of organisms is small. The details of the method may be seen in table 1. The test organism used is *Streptococcus hemolyticus*, strain C203MV (group A). The end point is read as the first tube in which there is no visible growth after incubation at 37 C for 24 to 30 hours. In experiments in which it was desired to avoid any appreciable amounts of serum in the assay, the same method was employed, except that the dilutions were made entirely in plain broth, and the culture was added in blood broth. The latter contained a suspension of defibrinated rabbit blood in an amount sufficient to give a final concentration of 2 per cent blood. In this type of experiment, the concentrations of penicillin were so chosen that the lowest levels would be in tubes containing not more than 1 to 2 per cent serum. The end point was read as the first tube in which there was no hemolysis or grossly visible growth. The broth was beef heart infusion broth, which contained 1 per cent peptone and 0.5 per cent NaCl, at pH 7.6. The cultures were 18-hour cultures of the organisms noted. The serum used routinely was pooled human serum heated to 56 C for 30 minutes and filtered through a Seitz filter. Each set of experiments was checked with fresh, sterile unheated serum.

The sensitivity tests were performed in the same way as the standard control in the assay method, and the sensitivity of the test organism was calculated as the concentration present in the tube at the end point. Variations in the final concentration of serum or albumin in the sensitivity tests were attained by appropriate variations in the concentration of these substances in the broth.

The following crystalline penicillins[2] were used: (1) benzylpenicillin (G); (2) *n*-heptylpenicillin (K); (3) *p*-hydroxybenzylpenicillin (X); and (4) *n*-amylpenicillin (dihydro F).

TABLE 1

Method of assay

DILUTION OF UNKNOWN (FINAL CONCENTRATION OF SERUM = 25 PER CENT)	1:120						1:20						1:4					
Ml of diluted unknown........	0.2	0.3	0.4	0.5	0.6	0.8	0.2	0.3	0.4	0.5	0.6	0.8	0.2	0.3	0.4	0.5	0.6	0.8
25 per cent pooled human serum in broth	0.6	0.5	0.4	0.3	0.2	0	0.6	0.5	0.4	0.3	0.2	0	0.6	0.5	0.4	0.3	0.2	0
Culture..	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Units per ml in unknown, when sensitivity of organism is 0.01 unit per ml	6	4	3	2.5	2.0	1.5	1.0	0.67	0.5	0.42	0.35	0.25	0.2	0.14	0.1	0.083	0.07	0.05

Procedure

I. Three dilutions of unknown serum are made as follows:
  (1) a 1:4 dilution in broth;
  (2) a 1:5 dilution of (1) using 25 per cent pooled human serum in broth as diluent;
  (3) a 1:6 dilution of (2), again diluting with 25 per cent serum.
  This gives final dilutions of 1:120, 1:20, and 1:4, each of which is 25 per cent serum.
II. Add the amounts of various dilutions as noted on table.
III. Make up each tube to volume of 0.8 ml with 25 per cent pooled serum.
IV. Add 0.2 ml culture to each tube. (Culture dilution = 2×10^{-5}.)
V. End point is read as first tube showing no growth after 24 to 30 hours' incubation A standard solution of the same penicillin in pooled serum is assayed as above. The sensitivity of the organism is calculated from this. The readings for any sensitivity are made conveniently by multiplying or dividing the reading given here.

Craig and his associates (1947) have recently demonstrated that significant amounts of as yet unidentified antibiotics were present in a sample of the crystalline sodium salt of penicillin K. Even if it be assumed that all samples of crystalline penicillin K consist of *n*-heptylpenicillin and unknown antibiotics, the biologic and pharmacologic behavior of such crystalline material appears to be sufficiently distinctive to warrant the use of the term K or preferably "K-type" penicillins. In order to simplify the subsequent discussion, the term K is used to designate crystalline material which consists of *n*-heptylpenicillin and possibly significant amounts of as yet unidentified "penicillins."

In the dialysis experiments, a cellophane bag, which contained 5 ml of pooled human serum or protein solution, was suspended in 20 to 100 ml of phosphate buffer (pH 7.38) containing 2 to 5 units of penicillin per ml. Dialysis was conducted for 48 hours at 10 C or for 4 hours in a shaking machine at room temperature. In some experiments, a second bag of serum containing 10 or 20 units per ml of the same penicillin was added to the flask in order to determine that equilibrium had been reached. It was assumed that this had occurred if the penicillin concentrations in the two bags were the same at the end of the experiment. A further check was made by determining the time at which the concentration inside the bag stopped rising.

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[2] The penicillins G, K, and dihydro F were obtained from Chas. Pfizer and Company. The penicillin X was obtained from Lederle Laboratories.

RESULTS

Effects of serum on the sensitivity of Streptococcus hemolyticus to individual penicillins. A series of sensitivity tests were performed with the standard test organism C203MV in media which contained various concentrations of pooled human serum. The basic medium was 2 per cent blood broth, and the final concentrations of serum were 10, 20, and 30 per cent, respectively. The concentration of penicillin required to prevent growth of the organisms in 2 per cent blood broth was compared with the concentration required in the presence of 10 to 30 per cent serum. The percentage of efficiency of the penicillin was then calculated as follows:

$$\frac{\text{Sensitivity of organism in 2 per cent blood broth}}{\text{Sensitivity in 2 per cent blood broth plus serum}} \times 100$$

The calculation was made to eliminate the day to day variations which always occur in a bioassay, to make the values comparable on either a unitage or a gravimetric basis, and to eliminate differences in the sensitivity of the particular organism to the individual penicillins. Thus a decrease in percentage of efficiency represents a decrease in the penicillin sensitivity of the organism, i.e., an increase in the concentration of penicillin necessary for the prevention of growth. As may be seen in figure 1, with the addition of increasing amounts of serum to the medium, there is a progressive decrease in the sensitivity of the test organism. The changes produced by the addition of serum are of the same degree for penicillins X, G, and dihydro F. The average efficiency of these three penicillins in 30 per cent serum was 36, 41, and 43 per cent, respectively. In contrast, the effect of serum to penicillin K was of an entirely different magnitude. The average efficiency of penicillin K in 30 per cent serum was only 7 per cent.

In analysis of the serum effect, it is necessary to consider three factors which might influence the results: (1) enhancement or inhibition of growth of the organism by the serum; (2) destruction of the penicillin by the serum during the incubation necessary for the bioassay; and (3) inactivation of the penicillin by binding to some constituent of the serum. Accordingly, the possible operation of these factors was investigated.

Effect of serum on the organism. The highest concentration of serum used in the sensitivity tests was 30 per cent. Higher concentrations of serum are known to exert an inhibitory effect upon the growth of the particular test organism used (Elias *et al.*, 1945). As may be seen in figure 2, however, the growth curves observed after the use of large and small inocula of C203MV were identical in blood broth and in the concentrations of serum which were used in the present experiments. In control experiments, it was established that the rise in pH produced by the addition of serum did not alter the results.

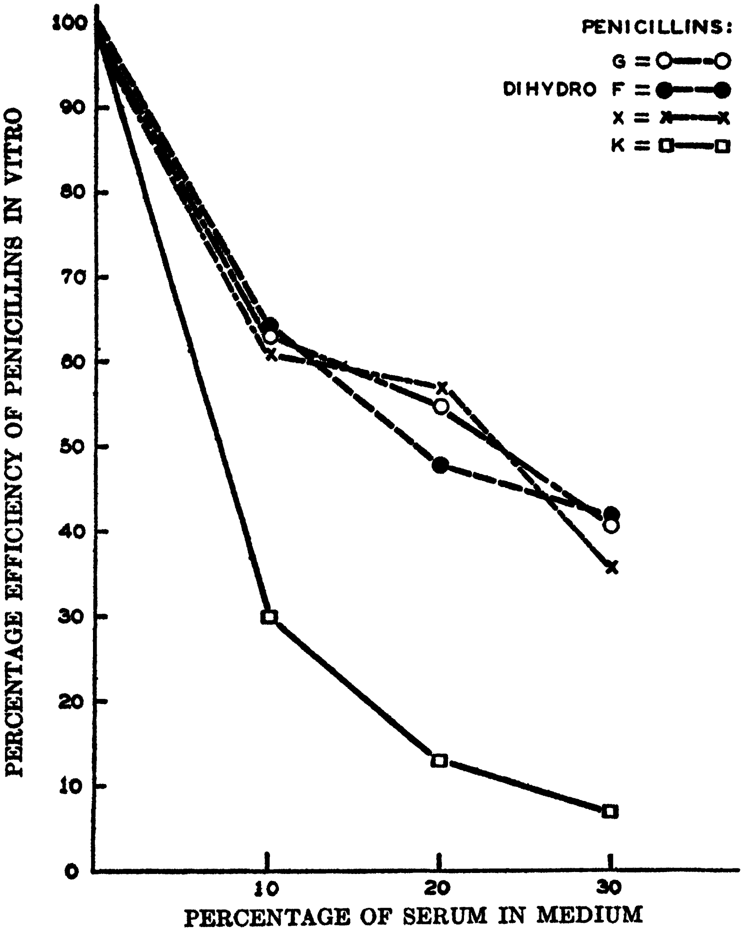

FIG. 1. EFFECT OF SERUM ON EFFICIENCY OF FOUR PENICILLINS

Destruction of penicillin by incubation in serum. Known amounts of the crystalline penicillins were added to serum, and the mixture was incubated at 37 C for 24 hours. Aliquots were removed for assay immediately after addition of the penicillin and at intervals thereafter. All samples were stored at −20 C until the completion of an experiment and were then assayed at the same time. Most of the experiments were performed with penicillins G and K.

Fresh human serum was collected from 4 to 6 persons, pooled, and used immediately. Parallel experiments were made with this and with a portion of the same serum heated at 56 C for ½ hour. In addition, heated, pooled human serum which had been filtered through a Seitz filter was used.

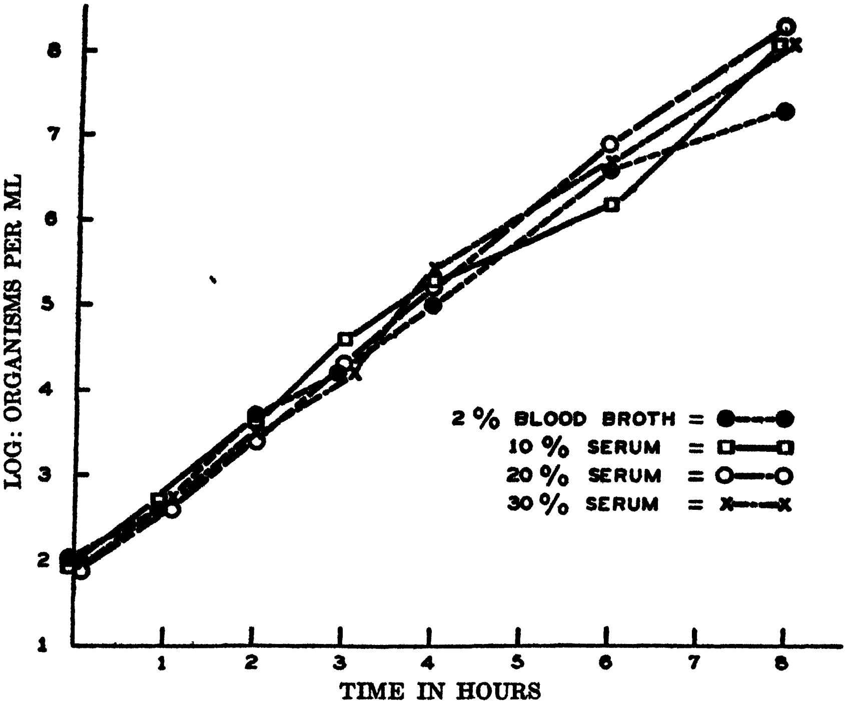

FIG. 2. GROWTH OF STREPTOCOCCUS HEMOLYTICUS (C203MV) IN THE TEST MEDIA

TABLE 2

Incubation of penicillins G and K in human serum

PENICILLIN	HOURS OF INCUBATION AT 37 C	PER CENT RESIDUAL		
		Average*	Minimum	Maximum
G	2	95	50	100
	4	92	50	100
	6	72	44	100
	12	35	25	65
	24	7	3	12.5
K	2	92	67	100
	4	91	65	100
	6	53	32	67
	12	29	25	33
	24		<3	4

* These values represent 12 experiments with G and 8 with K.

In the majority of the experiments, the initial concentration of penicillin was 10 units per ml of 98 to 99 per cent serum. In other experiments, 0.2 to 0.5

units of penicillin per ml were incubated in 98, 30, 20, and 10 per cent serum, which was diluted in the broth used in the sensitivity test. The results may be summarized as follows (table 2): (1) Penicillins G and K, are inactivated under the conditions described. (2) Both are inactivated from 90 to 100 per cent in 24 hours by whole serum, whether it be heated or unheated.

The inactivation does not appear to be a first-order reaction. With rare exceptions, no inactivation was apparent after 2 hours' incubation. Inactivation usually appeared after 4 to 6 hours' incubation of penicillin G and after 2 to 4 hours' incubation of penicillin K. Once their action appeared, it proceeded at a fairly regular rate throughout the 24-hour period. It should be emphasized that in this type of experiment the behavior of the same preparations of penicillins G and K varies widely from day to day. In any individual experiment, however, in which penicillins G and K were compared simultaneously under identical conditions, the onset of inactivation consistently appeared earlier with penicillin K than with penicillin G. It was not possible to demonstrate that the rate or the onset of inactivation of penicillins G and K was any different in heated than in unheated serum. In the experiments in which low concentrations of penicillins G and K (0.2 to 0.5 units per ml) were incubated with 10, 20, and 30 per cent serum in broth, no inactivation of the penicillins appeared during a 12-hour period of observation. The concentrations of penicillins and serum used in the latter experiments are comparable to those usually present during the bioassay of specimens of serum obtained from a penicillin-treated subject.

TABLE 3

Results of a typical dialysis experiment

PENICILLIN	SERUM UNITS PER ML	DIALYZATE UNITS PER ML
X	2.5	1.5
G	2.5	1.0
Dihydro F	4.0	1.3
K	15.0	0.5

TABLE 4

Binding of penicillin to human serum and purified plasma fractions

PENICILLIN	AVERAGE PER CENT BOUND				
	Human serum	Human albumin Fraction V	Bovine albumin	Human plasma Fractions I, II, III, IV-1, IV-1,1, IV-4	Bovine plasma Fraction II
X	47		53		0
G	58	60	52	0	0
Dihydro F	63		66		0
K	91	86	86	0	0

Binding of penicillin to serum. The results of the dialysis experiments may be seen in tables 3 and 4. Each value in table 4 represents the average of

6 to 8 dialysis experiments with the particular penicillin. The variation between individual experiments was slight. As may be seen, there was a significant degree of binding of all of the penicillins to some constituent of serum. Penicillin X was bound the least (47 per cent). Penicillins G and dihydro F were bound to a higher degree, i.e., 58 and 63 per cent, respectively. In contrast to these values, the degree of binding of penicillin K was 91 per cent, or almost double the average value for penicillin X. As may also be seen in table 4, in each instance the degree of binding of an individual penicillin to serum and to a solution of 5 per cent bovine albumin was of the same order of magnitude. The binding of all four penicillins to bovine globulin (fraction II)[8] was investigated. In addition, penicillins G and K were dialysed against fractions I,

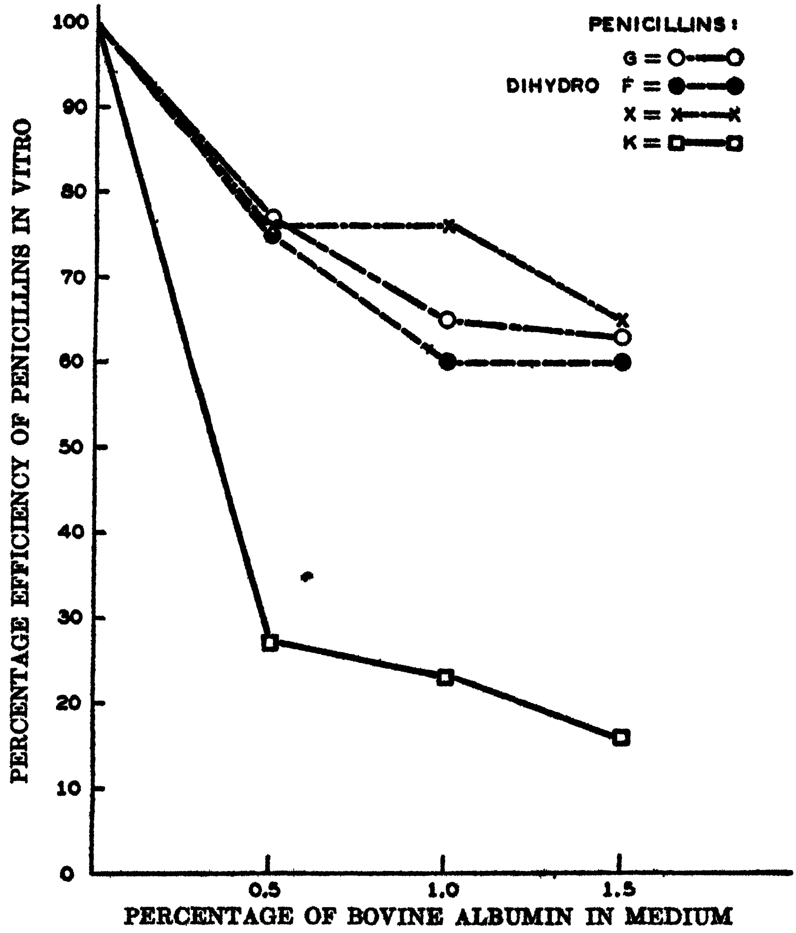

FIG. 3. EFFECT OF ALBUMIN ON EFFICIENCY OF FOUR PENICILLINS

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[8] The bovine globulin was obtained through the courtesy of Mr. E. B. Lesh, Armour Laboratories, Chicago, Illinois.

II, III, IV-1, IV-1, 1, IV-4, and V of human plasma.[4] The concentrations of the individual protein fractions approximated those usually present in plasma. As may be seen (table 4), the degree of binding of penicillins G and K to human fraction V closely approximated the degree of binding of these penicillins to bovine albumin and human serum. In contrast, neither penicillin was bound to any of the other protein fractions of plasma.

Effect of albumin on the sensitivity test. The effect of bovine albumin on the sensitivity of hemolytic streptococci to the four penicillins was investigated. The concentrations of albumin in the media were comparable to those attained by the addition of 10, 20, and 30 per cent serum. As may be seen in figure 3, the effect of bovine albumin on the efficiency of the penicillin was similar to that of human serum. As with serum, the albumin effect was most pronounced with penicillin K, but was significant with all of the penicillins tested. The factor of pH was controlled as in the previous experiments. The addition of 1.0 per cent of bovine albumin did not alter the growth curve of the organism (figure 4). Comparison of the data in figures 1 and 3 indicates that, although the effect of albumin parallels that of serum, the latter produces an effect of greater magnitude. This suggests that other factors may also be present.

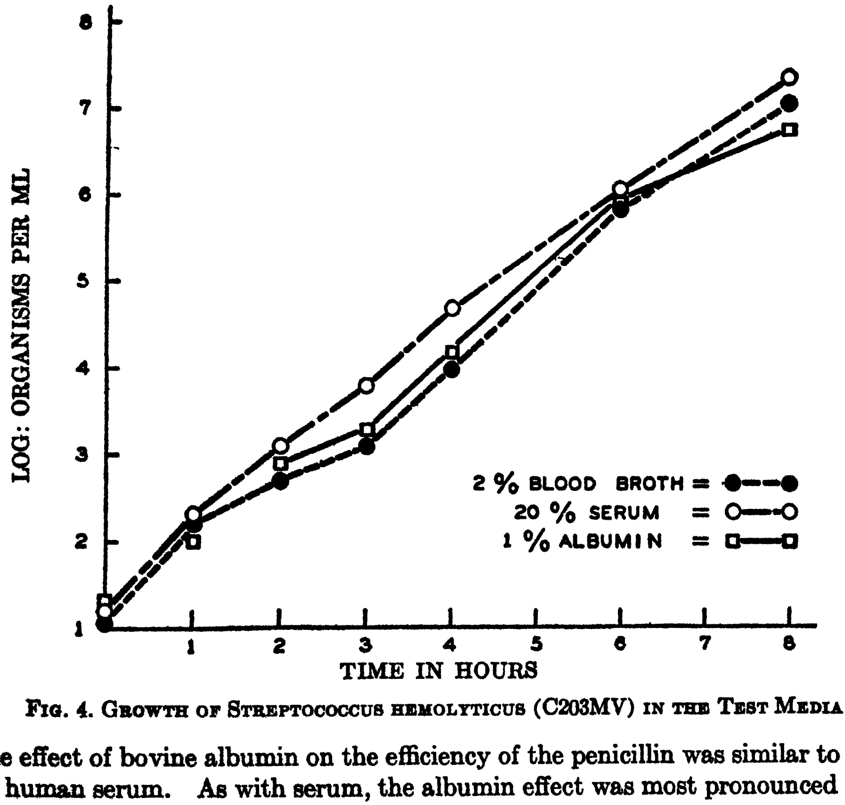

FIG. 4. GROWTH OF STREPTOCOCCUS HEMOLYTICUS (C203MV) IN THE TEST MEDIA

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[4] The fractions of human plasma were obtained through the courtesy of Dr. S. Howard Armstrong, Jr., Harvard University Medical College.

Sensitivity tests with pneumococcus and Staphylococcus aureus. In order to exclude the possibility that the results observed were caused by factors unique to the particular test organism used, similar experiments were performed with other organisms. Pneumococcus type II (D-39) and *Staphylococcus aureus* (Oxford H strain) were the organisms tested. The results are presented in table 5. As may be seen, with these organisms also, the presence of serum decreased the efficiency of all of the penicillins, and the effect was most pronounced with penicillin K.

TABLE 5

Comparative efficiency of penicillins against Staphylococcus and pneumococcus

TEST ORGANISM	PENICILLIN	EFFICIENCY OF PENICILLIN IN 30 PER CENT SERUM
		%
Staphylococcus aureus	X	67
	G	57
	K	5
Pneumococcus type II	X	39
	G	40
	K	9

DISCUSSION

On the basis of these observations, it appears that (1) the antibacterial activities of four penicillins were antagonized *in vitro* by serum and by the albumin fraction of serum; (2) the degrees of antagonism were quantitatively different for the individual penicillins; and (3) among these four penicillins, the degrees of reduction in antibacterial activity caused by serum and albumin were roughly in direct proportion to the degrees of binding to these substances as demonstrated by dialysis.

The most satisfactory rationalization of these data is that the individual penicillins bind *in vitro* to a varying degree to the albumin component of serum, and that the resulting penicillin-albumin complex has little or no antibacterial activity. Investigation of alternative explanations for the observed phenomena have been made. The growth curve of the test organism in 2 per cent blood broth was not altered by the addition of 10 to 30 per cent serum or 1.0 per cent bovine albumin (figures 2 and 4). The possibility that serum or albumin may increase the penicillin resistance of bacteria without affecting their growth characteristics cannot, however, be explicitly excluded.

Although inactivation of the penicillins occurred after prolonged incubation in serum, it does not appear that this reaction is an explanation for the decreased activity of these drugs in the presence of serum or albumin. This type of inactivation during incubation did not usually start until after 2 to 4 hours' incubation, a time at which much of the effect of the penicillin upon the organisms would presumably have already occurred. Moreover, inactivation of penicillin resulting from incubation in serum was notably irregular, in contrast to the serum effect upon the penicillin sensitivity of the organisms, which was regular and readily reproducible. The uniformity of the antagonistic effect of serum on an individual penicillin, coupled with the marked difference among the different penicillins, makes it seem unlikely that the effect is a result of destruction of penicillin by the incubation during the bioassay procedure.

It should be noted that important differences exist between the conditions which obtain *in vivo* and the conditions of penicillin-serum incubation experiments such as those presented above. The stability of all of the penicillins is considerably different at various levels of pH (Benedict *et al.*, 1946), and the pH of human serum *in vitro* is 8.0 or higher. Moreover, the delay in the onset of inactivation which occurs on incubation *in vitro* strongly suggests that the inactivating substance is not normally present in serum but is produced artificially by the procedure.

If protein-bound penicillin is inactive, it would be anticipated that a penicillin that is bound to a high degree would be considerably less efficient in the presence of serum or albumin than a penicillin that is bound to a lesser degree. In the present experiments, penicillin K, approximately 90 per cent of which is bound to serum or albumin, lost 85 to 90 per cent of its activity *in vitro* against the test organism in the presence of 30 per cent serum or 1.5 per cent albumin. In contrast, under the same conditions penicillin X, which binds much less (47 per cent), lost only 40 to 60 per cent of its activity.

It has not been possible, as yet, to determine by direct observation whether the penicillin-albumin complex exerts antibacterial action *in vitro* or *in vivo*. The reason for this is that the degree of binding of penicillin to albumin, like that demonstrated for other drugs (Davis, 1946), presumably represents a balance between the concentrations of bound and unbound drug present in the system. The degree of this type of binding is readily altered by dilution. Therefore, measurement of the activity of the penicillin-albumin complex by bioassay or after parenteral administration, both of which necessarily involve dilution, cannot represent direct observation of the activity of the original material before dilution.

Despite the absence of direct proof, however, it would seem that the protein-bound portion of the penicillin is inactive *in vitro*, from the facts that each penicillin is bound to albumin or serum to a certain degree; that the degree of binding and the loss of antibacterial activity in protein solutions are constant and are unique for each penicillin; and that the loss of antibacterial activity in protein solutions does not appear to result from penicillin destruction or from action of the protein on bacterial growth.

Whether penicillin is bound to albumin *in vivo* to the same extent as occurs *in vitro* has not been determined for the reasons noted previously. There is evidence which suggests, however, that the binding of penicillin to protein *in vivo* does occur. There is a striking parallelism between the efficiency of a particular penicillin when tested *in vitro* in the presence of albumin and its therapeutic effectiveness *in vivo*. The *in vitro* activity of penicillin X is the least affected by serum or albumin, and the drug binds to albumin to the least degree of any of the penicillins tested. In experimental infections *in vivo*, penicillin X is the most active of these four penicillins (Eagle and Musselman, 1946; Hobby *et al.*, 1946). In contrast, the *in vitro* activity of penicillin K is considerably reduced by serum and albumin, the drug binds to albumin to a high degree and is the least active of these four penicillins when tested *in vivo*. Thus the apparent disparity between the *in vitro* and the *in vivo* activity of these four penicillins disappears when the *in vitro* testing is performed in the presence of serum or albumin.

These results are essentially the same as the results previously observed in similar experiments with the sulfonamides. In both instances, all of the compounds studied were bound to albumin to a significant degree. With the sulfonamide drugs, it was reported (Davis and Wood, 1942) that the degree of binding parallels the *in vitro* activity when the latter is determined in the absence of protein. In the present investigation, a similar relationship was observed. In the absence of protein, penicillin X is the least, and penicillin K the most, active of the four compounds.

If the penicillins are bound to the plasma albumin *in vivo*, it would be anticipated from the experience with other albumin-bound agents that the degree of binding of a particular penicillin would exert a considerable influence on its distribution, conservation, or excretion by the kidneys, and ultimate fate. Moreover, as suggested for the sulfonamides by Davis (1943, 1946), appreciably lower concentrations of penicillin would be necessary for therapeutic effectiveness in fluids which are poor in protein, such as the cerebrospinal fluid, than would be required in the blood or the extracellular fluid in general. Possible support for the latter notion is afforded by the excellent results which have been observed in the treatment of neurosyphilis following the administration of penicillin solely by the intramuscular route. On the regimens which have been therapeutically successful, only minute amounts of penicillin appear in the cerebrospinal fluid even after 2 weeks of continuous therapy. The penicillin concentrations in the interstitial fluid of the brain are presumably equally low, for both fluids eventually attain equilibrium. Despite the low concentrations of penicillin attained in these protein-poor fluids, the therapeutic action of the drug is striking.

Regardless of the occurrence of binding to protein *in vivo*, the effects observed are important in studies of the absorption and distribution of the individual penicillins in man. The numerous disadvantages of the bioassay of penicillin are greatly multiplied by lack of satisfactory controls. The phenomena observed here interfere with the bioassay of all four penicillins in specimens of serum, and the effect is quantitatively different in each.

The conventional bioassay consists of (1) a stepwise dilution of the unknown specimen into a series of tubes, and (2) the addition of broth and culture to the tubes, bringing all tubes to a constant volume. The unknown specimen is then compared with a specimen of known concentration, and the penicillin sensitivity of the organism is calculated from this standard. A direct calculation is then made from standard to unknown, *using only the dilution of the penicillin* in the calculation.

As we have seen, the penicillin sensitivity is different in different concentrations of serum. As each tube in the lower range of the test contains a different concentration of serum, the penicillin sensitivity is different in each tube. Consequently, an end point in the lower range is directly comparable *only* with a standard which contains the same concentration of serum, and is not directly comparable with another concentration of penicillin in serum, or with any standard which does not contain serum. It should be emphasized that this factor is not controlled simply by starting with a standard which is in serum. To correct this error, one may maintain a constant percentage of serum in each tube by the addition of pooled serum. This allows a direct comparison of unknown to standard. By this method, using *Streptococcus hemolyticus* C203MV as the test organism, the lowest concentration of the four penicillins which can be measured in an unknown serum varies from 0.05 unit per ml of penicillin X to 0.4 to 0.5 unit per ml of penicillin K.

Following the parenteral administration to an adult of 20,000 to 90,000 units of any of the four penicillins used in the present study, the concentration in the serum would usually fall below 0.3 unit per ml during the first hour after injection of the drug. As it is impossible to measure penicillin K at this concentration in serum, the false impression would be created that the penicillin K had "disappeared" from the blood within 30 to 60 minutes. In contrast, penicillins G and X, which can be measured in appreciably lower concentrations, would be detectable for much longer periods of time after the parenteral injection.

It appears, therefore, that the rapid "disappearance" of penicillin K from the blood of humans, noted in this and in other laboratories, is in all likelihood an artifact attributable to the marked effects of serum (presumably albumin binding) upon the activity of penicillin K in the *in vitro* assay. It should not be inferred from these observations that the stability of penicillin K within the body is necessarily the same as that of the other penicillins. On the contrary, if the same high degree of binding to albumin occurs *in vivo* as *in vitro*, it is conceivable that the rate of excretion through the kidney could be sufficiently slowed and the distribution in the tissues altered so that more of this penicillin per dose would be exposed to destructive influences within the body. It might be possible, by the use of much larger doses than have hitherto been employed, to obtain a more accurate estimation of the comparative rates of disappearance of the individual penicillins from the circulating blood. An investigation of this aspect of the subject is in progress.

As the effect of serum upon the sensitivity of the bioassay may vary so considerably among individual penicillins, the magnitude of the effect for each newly introduced penicillin must be determined before its distribution within the body can be properly studied. Moreover, the apparent correlation between the binding of penicillin to albumin *in vitro* and the therapeutic effectiveness of the drug *in vivo* suggests that this procedure should also be applied to all new penicillins.

SUMMARY

It was observed that the antibacterial activities of four penicillins, X, G, dihydro F, and K, were antagonized *in vitro* by serum and by the albumin fraction of serum.

The degrees of antagonism were quantitatively different for the individual penicillins.

Among these four penicillins, the degrees of reduction in antibacterial activity caused by serum and albumin were roughly in direct proportion to the degrees of binding to these substances as demonstrated by dialysis. Penicillin X, which was 47 per cent bound, lost 40 to 60 per cent of its activity in the presence of 30 per cent serum. Penicillin K, which was 91 per cent bound, lost 85 to 90 per cent of its activity when similarly tested.

There is an inverse relationship between the degree of binding of the individual penicillins to serum *in vitro* and the reported effectiveness of these penicillins in the treatment of infections in experimental animals.

ADDENDUM

Subsequent to the preparation of this manuscript, a study of the distribution of penicillins G and K after intravenous administration in dogs was reported by Richardson and his associates (Proc. Soc. Exptl. Biol. Med., 1946, **63**, 514). Although different techniques were employed, certain observations were made which are in agreement with those reported here. These investigators observed that in the cup test assay it was not possible to obtain complete recoveries of penicillins G and K added to plasma. The recovery of K was considerably lower than that of G. Evidence was presented that the low recovery was not due to destruction of the penicillin. It was also observed in dialysis experiments that penicillin K was bound by plasma to a higher degree than penicillin G.

Thus it would appear that, insofar as they are parallel, the observations reported here confirm those of Richardson and his coworkers.

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A STUDY OF SENSITIVITY TO HEMOPHILUS PERTUSSIS IN LABORATORY ANIMALS

I. The Hypersensitivity of Laboratory Animals to Hemophilus pertussis

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The hypersensitivity of laboratory animals to *Hemophilus pertussis* has been challenged in several publications that will be discussed below. We considered this problem worthy of special investigation. We studied the sensitivity of mice, guinea pigs, and rabbits to *H. pertussis*. For the experiments described in the three following papers, we used more than 700 mice, 100 guinea pigs, and several dozen rabbits. Our studies along this line began with experiments on the sensitivity of mice and guinea pigs to filtrates of *H. pertussis* culture.

The filtrates of *H. pertussis* culture were obtained in the manner described by Roberts and Ospeck (1942, 1944). These freshly prepared filtrates were toxic for mice, the lethal dose being 0.05 to 0.02 ml.

In order to obtain sensitizing material in more concentrated form fractionation with ammonium sulfate was performed. Six hundred grams of dry ammonium sulfate were added per 1 liter of filtrate. The resulting precipitate was resuspended in a small volume of water and dialyzed; following dialysis the material was dried by lyophilization. For our experiments a 5 per cent solution was prepared from the dry powder. This material will henceforth be designated as AF (allergenic fraction). In most cases this fraction was found to be nontoxic for normal mice and was used for sensitization. However, some batches contained traces of a toxic material and upon injection killed some of the mice. Detoxification of such batches by the addition of 0.3 per cent formalin and incubation for 1 to 3 days at 37 C did not destroy their sensitizing property. A fraction from broth medium was prepared in the same way for use as a control and will be designated below as BF (broth fraction).

EXPERIMENTS WITH MICE

Mice were very easily sensitized from a single intra-abdominal injection of 0.25 to 0.5 ml of AF (5 per cent solution). This solution always contained some insoluble matter; however, for sensitization the whole mixture was injected intra-abdominally. Seven to ten days after the first injection the mice were tested for sensitivity by an intravenous injection of the clear supernatant of a 5 per cent solution of AF. All sensitized mice could be killed by this injection, and the lethal dose varied from 0.05 to 0.1 ml. Normal mice tolerated 0.5 ml of the same material.

Special experiments were performed to determine whether sensitization was due to products of *H. pertussis* organisms or to the broth. Mice sensitized with AF were injected intravenously with BF (prepared by ammonium sulfate precipitation of broth). Survival of almost all of the sensitized mice after injection of the broth fraction indicated that sensitization was induced by materials elaborated by the *H. pertussis* organism. Table 1 illustrates one of many similar experiments.

In the course of this work we also studied the susceptibility of normal and sensitized mice to unconcentrated filtrate of *H. pertussis* culture. Some of these filtrates were detoxified with formalin (toxoid), and others were boiled. Several batches of these materials were injected simultaneously into normal and sensitized mice, and the results are recorded in table 2.

By referring to table 2 it can be seen that the allergenic property of *H. pertussis* filtrate is heat-stable, but the toxic principle is heat-labile. Detoxification with formalin reduces the toxicity for normal mice but has no influence on the allergenic effect for sensitized mice. Apparently the toxic filtrate of *H. pertussis* culture used in these experiments contained about five anaphylactic units which could not be destroyed or inactivated by the addition of formalin or heating. The higher toxicity of *H. pertussis* filtrate to sensitized mice as compared with normal mice might depend on the combined action on sensitized mice of the toxic principle together with traces of allergenic material, since in these experiments we injected a volume of toxin that did not contain enough allergenic principle to produce shock by itself in sensitized mice (less than 0.2 ml). The sensitized mice died 10 minutes to several hours after intravenous injections of AF, depending on the dosage. Injection of AF into sensitized mice caused difficulties in respiration.

The sensitization described persists for several months following a single injection of AF.

TABLE 1

Test of sensitized and normal mice with AF and BF

TEST MATERIAL DOSE PER MOUSE (INTRAVENOUS)	TEST MATERIAL AF		TEST MATERIAL BF	
	Sensitized mice	Normal mice	Sensitized mice	Normal mice
ml				
0.5		2/2		
0.4		2/2		2/2
0.3		2/2	4/5	4/4
0.2		2/2	9/10	4/4
0.1	0/6*	2/2	4/4	2/2
0.05	0/9			
0.02	1/2			

* In all our tables the total number of mice is shown in the denominator and the number of survivals in the numerator.

EXPERIMENTS WITH GUINEA PIGS

We continued our experiments on sensitization by observing the allergenic effect of *H. pertussis* filtrate on guinea pigs. Some groups of guinea pigs were sensitized with unconcentrated *H. pertussis* filtrate detoxified with formalin. They received two 2-ml intramuscular injections, the second injection being administered 1 month after the first. Another group was sensitized by a single intra-abdominal injection of 2 ml of AF (that had not been incubated with formalin).

Three to four weeks later the guinea pigs were tested for sensitivity by an intracardial injection of the clear supernatant of AF. Thirteen (of 17) sensitized guinea pigs were injected with AF in amounts of 0.1 ml, 0.25 ml, 0.5 ml, and 1.0 ml per guinea pig. Of the 13 guinea pigs, 9 died, many with symptoms of anaphylactic shock. Death occurred within 10 to 20 minutes after injection (the minimal dose that produced shock was 0.25 ml of AF). Autopsy revealed emphysema of the lungs and enlarged heart. The other 4 sensitized guinea pigs were injected intracardially with 1 ml of BF; all survived.

TABLE 2

The susceptibility of normal and sensitized mice to H. pertussis toxin and toxoid

DOSE OF TOXIN OR TOXOID INJECTED INTRAV. PER MOUSE	SENSITIZED MICE	NORMAL MICE
Toxin boiled before injection		
ml		
0.4	0/2	4/4
0.2	0/4	4/4
0.1	2/2	
Toxin not boiled		
0.1	0/6	0/12
0.05	1/8	2/16
0.02	1/12	14/18
0.01	2/2	2/2
Formol toxoid		
0.4	0/2	2/2
0.2	0/4	2/2
0.1	2/2	

Nine normal guinea pigs of similar weight were used as controls. These were injected with 0.5 ml and 1.0 ml of the AF. Only 1 of the 9 normal guinea pigs died (from 0.5 ml).

DISCUSSION

Hypersensitivity of laboratory animals to *H. pertussis* has been discussed by numerous authors. Several observers have noticed that, during immunization with *H. pertussis* antigens, laboratory animals developed sensitivity to this material.

Ospeck and Roberts (1944) stated that attempts to immunize mice actively with H. *pertussis* toxoid and with sublethal doses of *H. pertussis* toxin as a whole gave discouraging results; they believe that those results may have been due to sensitization. Sprunt and Martin (1943) studied the lesions produced in the lungs after intratracheal injection of *H. pertussis* toxin into rabbits that had previously been inoculated intravenously with different amounts of antitoxin. They noticed that rabbits which had received the larger doses of antitoxin had larger lesions, and they attributed the cause in part to an allergic reaction due to passive sensitization to some other antigenic substance present in the toxin. Felton and Ottinger (1942) observed that a modifying dose of antigen, polysaccharide, or vaccine could interfere with the development of immunity in mice against pneumococci. For instance, injection of 0.5 mg of polysaccharide into mice paralyzed the development of immunity by subsequent injections of immunizing doses of the same antigen. G. Eldering (1942) reported that injections of the polysaccharide of *H. pertussis* made mice more susceptible to injections of living *H. pertussis*. Polysaccharide for these experiments was extracted from the *H. pertussis* organism in accordance with the methods of Boivin *et al.* (1933) and Felton and Kauffmann (1938).

We found the *H. pertussis* toxic filtrates to contain substances precipitable with specific antibacterial serum. In our previous work with diphtheria toxin (Parfentjev *et al.*, 1942), we were able to remove, by absorption with magnesium hydroxide, a fraction containing bacterial antigens without greatly impairing the potency of the toxin. Fractionation of *H. pertussis* filtrate, however, presented special difficulties due to the low initial titer and instability of its toxic principle.

H. pertussis toxic filtrates represent complex mixtures of different components, the biological significance of which is not clear. This might be responsible to a great extent for the conflicting results obtained by different authors who used this material for testing cutaneous sensitivity of laboratory animals and human beings to *H. pertussis* (Lapin 1942, 1943). Considerable progress in this respect was achieved by Flosdorf and his collaborators (1940, 1941, 1942, 1943) and by Smolens and Mudd (1943), who introduced purified agglutinogen for the cutaneous tests. Strean (1940) described necrosis after intracutaneous injection into normal rabbits of crude or purified (by precipitation with acid) *H. pertussis* endotoxin. In our present work with laboratory animals we found the technique outlined convenient for the study of bacterial allergy pertaining to *H. pertussis*.

SUMMARY

Manifestations of experimental allergy in laboratory animals to soluble substances of *Hemophilus pertussis* toxic filtrates have been demonstrated.

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A STUDY OF SENSITIVITY TO HEMOPHILUS PERTUSSIS IN LABORATORY ANIMALS

II. Hemophilus pertussis Allergen and Its Assay on Laboratory Animals

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This paper deals with our attempt to isolate different fractions from *Hemophilus pertussis* organisms and to estimate their influence on the development of sensitivity. It is also concerned with methods of assaying *H. pertussis* allergen.

THE PREPARATION OF FRACTIONS FROM H. PERTUSSIS

The *H. pertussis* organisms used for this work were grown on blood agar or in liquid media; however, no difference was noted in the final products derived from organisms grown in these media. In the course of this work we prepared several fractions from *H. pertussis* organisms.

One of these fractions was a denaturated nucleoprotein which we prepared by first extracting lipoids from the organisms, following the technique of R. J. Anderson (1927*a*, 1927*b*, 1932). According to this method, the bacilli collected at the end of the growth period were first dehydrated with acetone and then extracted with a mixture consisting of equal parts of alcohol and ether containing 1 per cent hydrochloric acid. After removal of the lipoid fraction the organisms were extracted twice with water acidified to pH 4. In this way proteins soluble in slightly acid condition were eliminated. Finally, the bacilli were extracted twice with water adjusted by the addition of sodium hydroxide to an alkaline reaction (red to phenolphthalein) and preserved with merthiolate 1:10,000. The extraction was continued until neutralization of the sodium hydroxide ceased. At the end of this period the liquid separated by centrifugation was Berkefeld-filtered.

By following this procedure we were able to prepare a dilute solution of *H. pertussis* nucleoprotein, which we concentrated by salting out with 600 grams of ammonium sulfate per liter of filtrate. The resulting precipitate was removed by filtration, redissolved in a small volume of water, dialyzed, preserved with merthiolate (1:10,000), and Berkefeld-filtered.

Chemical tests performed with this material indicated a complex protein containing a nucleoprotein group. This protein complex is soluble in water at a neutral or alkaline reaction, is nondialyzable, and is insoluble in 60 per cent ammonium sulfate. It is heat-coagulable and its isoelectric point is near pH 4. In the absence of sodium chloride it can be purified by repeated precipitation with acid at pH 4. Solutions of this nucleoprotein so purified were used for the chemical tests.

The material gave positive biuret, xanthoprotein, Millon, Hopkins-Cole, and Sakaguchi (Weber, 1930) tests, the last one reacting positively when an amount of protein containing 0.5 mg nitrogen was used. The diphenylamine test of Dische for nucleoprotein, modified by Thomas (1931), Seibert (1940), and Dische (1944), gave a strong positive reaction with a solution of this protein containing 0.5 mg or less of nitrogen. The carbazole test described by Gurin and Hood (1939, 1941) also gave a strong positive reaction in concentrations of 0.05 mg nitrogen or less. (In our work we found it more convenient to use 0.25 per cent carbazole than 0.5 per cent.) After a portion of nucleoprotein was ashed with a mixture of sulfuric and nitric acids, the hydrolyzate reacted positively with molybdate solution and magnesia mixture, indicating phosphate.

We also performed partial hydrolysis of the nucleoprotein by heating it with 5 per cent sulfuric acid for 1 hour on a boiling water bath. At the end of this time the material was neutralized with ammonium hydroxide, centrifuged, and the supernatant submitted to several tests. Both the Molisch test for carbohydrate and the Bial test for arabinose were positive. The presence of purine base was established by positive reactions with ammoniacal silver nitrate and with copper sulfate and sodium bisulfite. Owing to the scarcity of the material, individual purine and pyrimidine bases could not be identified. Analysis of a few batches of nucleoprotein showed the values for nitrogen and phosphorus to be 12 to 15 per cent and 4 to 6 per cent, respectively.

This nucleic acid protein complex—a designation used by Mirsky (1943)—produced anaphylactic shock when injected into sensitized animals, and will be referred to as NPD (nucleoprotein denatured). As was pointed out, our product (NPD), owing to its treatment with strong hydrochloric acid for removal of lipoids followed by prolonged alkali extraction, contains denatured proteins, as indicated by its physical properties. A solution of NPD is very transparent, has very low viscosity, and is not precipitable with 0.14 molar sodium chloride or 0.1 per cent calcium chloride, as is characteristic of some undenatured nucleoproteins; for example, those described by Mirsky and Pollister (1943) for fibrous nucleoprotein from chromatin, and by Hall (1941) for thymus nucleohistone.

By adapting the technique of Hall, we were able to prepare undenaturated nucleoprotein from *H. pertussis* organisms. However, because of its high viscosity and poor solubility this material presented great difficulties for animal experimentation. For this reason, most of our animal tests were conducted with NPD.

We also performed some experiments on the isolation of nucleic acid from NPD. For this purpose NPD was broken down by the following consecutive steps: (1) A solution of protein was diluted at neutrality with 0.85 per cent sodium chloride to 0.5 to 1 mg nitrogen per ml, and immersed in a boiling water bath 5 to 10 minutes. After cooling, the coagulated proteins were removed by centrifugation. (2) The supernatant was then acidified and the resulting precipitate of acid-insoluble proteins (PF) removed by centrifugation. (3) To the second supernatant, without neutralization, was added 20 per cent alcohol; the material was kept for 24 hours at 4 C, and at the end of this period was centrifuged at the same temperature to eliminate a small precipitate. (4) The final supernatant containing nucleic acid was adjusted to pH 4 by the addition of sodium hydroxide and mixed with an equal part of alcohol. At approximately 60 per cent alcohol we could precipitate nucleic acid free from protein. The nucleic acid (NA) obtained was redissolved to the original volume. The material gave a negative biuret test but positive Dische and Gurin tests. Quantitative determinations performed with the last two tests indicated that we recovered most of the carbohydrate and nucleic acid in this fraction. However, the fraction might still contain traces of protein in an amount not great enough to be detected by the biuret test. During the procedure described above, most of the proteins were collected in the first two fractions.

EXPERIMENTS WITH MICE

For the experiments described in this section, mice were sensitized by a single intra-abdominal injection of *H. pertussis* vaccine, the degree of sensitivity achieved depending on the amount of vaccine injected. In most cases, each mouse received 50 billion organisms killed by the addition of merthiolate (1:20,000) and 0.25 per cent phenol to *H. pertussis* culture. Such a dose contained 0.15 to 0.2 mg nitrogen. However, the injection of this amount of vaccine killed 20 to 25 per cent of the mice used in some experiments. We found that soaking the organisms for a day or two in chloroform considerably reduced the toxicity of the vaccine without impairing its sensitizing property. Doses of *H. pertussis* smaller than that cited above also induced a noticeable sensitivity in mice.

In contrast to this, the treatment of *H. pertussis* vaccine with a mixture of equal parts of alcohol and ether containing 1 per cent hydrochloric acid greatly reduced its sensitizing properties. In a number of experiments, injection of vaccine treated in this manner failed to produce sensitivity. According to our experiments, the injection of NPD even in massive doses did not produce anaphylactic sensitivity in mice. Table 1 presents data relating to the toxicity of NPD for normal animals as compared with sensitized mice (one experiment).

The lethal dose of NPD for normal mice is equal to 0.6 mg nitrogen per mouse or 30 mg nitrogen per kilogram of body weight. Sensitized mice succumbed from a dose $\frac{1}{30}$, or less, of that required to kill normal mice. In some individual experiments, the difference in the lethal dose for normal and sensitized mice was as great as 100 times. Death in sensitized mice occurred in from 10 to 20 minutes to several hours (12 to 16 hours) after injection, depending on the dose of NPD given. The material caused acceleration of the respiratory movement, which became increasingly difficult with the passage of time. Many mice had convulsions before death occurred. Similarly, we noticed that sensitized mice succumbed more readily than normal mice to intra-abdominal injections of *H. pertussis* vaccine in massive doses.

We also tested the susceptibility of mice sensitized with *H. pertussis* vaccine to different proteins. In the course of this work we prepared NPD from *Brucella abortus*, strain 19, and AF from the filtrate of *Brucella bronchiseptica* culture. Both these materials, together with commercial tuberculin, various sera, broth, etc., were used for testing sensitized mice. From these experiments we learned that mice sensitized with *H. pertussis* vaccine could be shocked with the proteins of *B. abortus* and *B. bronchiseptica*, but these mice did not exhibit any increase in susceptibility to injections of proteinic material in general, i.e., horse, rabbit, and guinea pig sera, commercial tuberculin, *H. pertussis* broth, etc.

Some attempts were made to determine which constituent of NPD produced shock in sensitized mice. For this purpose, fractions of NPD, described above, were tested on sensitive mice. The doses were figured in the amount of nitrogen per injection as well as the amount of nucleic acid that the mice received with these injections. As a standard for this test, we accepted the amount of nucleic acid contained in the original NPD, as determined with the Evelyn colorimeter by using the technique of Seibert (1940). The amount of nucleic acid found in the different fractions was referred to this standard. The results of these attempts appear in table 2.

TABLE 1

Injection of normal and sensitized mice with H. pertussis NPD

DOSE CALCULATED IN MG N PER MOUSE	RESULTS
Normal mice	
0.6–1.0	4/18*
0.2–0.5	39/50
Sensitized mice	
0.6	0/4
0.12	0/4
0.2	0/5
0.08	2/7
0.04	7/12
0.02	2/2

* In all the tables contained in this paper, the total number of mice is shown in the denominator and the number of survivals in the numerator.

According to table 2, the mice received in the preparation containing nucleic acid 5 times more nitrogen and 30 times more nucleic acid than was contained in the original NPD, and still all the mice survived. This indicated that the mice injected with *H. pertussis* antigens became sensitive to the protein portion of nucleoprotein, but not to the nucleic acid.

EXPERIMENTS WITH GUINEA PIGS

Toxicity of H. pertussis protein for guinea pigs. The toxicity of *H. pertussis* NPD was studied with normal and sensitized guinea pigs by intracardial injection of solutions containing different concentrations of the materials. Sensitized guinea pigs were prepared by a single intra-abdominal injection of *H. pertussis* AF or vaccine administered about two months before this test was performed. Insofar as the results obtained with guinea pigs sensitized with both materials were the same, the data of these tests were combined and tabulated against the results of experiments with normal guinea pigs. The guinea pigs used in one of these tests (table 3) had an average body weight of about 400 grams. The dose injected was calculated in mg of nitrogen of *H. pertussis* NPD per kilogram of body weight of guinea pigs.

Injections of lethal or sublethal doses of NPD into guinea pigs (normal and sensitized) at first greatly accelerates the respiratory movements of the animals. In animals receiving lethal doses of *H. pertussis* protein (NPD) the difficulties in respiration become aggravated with the passage of time. Sensitized guinea pigs injected with the same material develop abundant exudate from the eyes and nose.

TABLE 2

Sensitivity of mice to different constituents of H. pertussis nucleoprotein

FRACTIONS*	AMOUNT N INJECTED PER MOUSE	MORTALITY OF MICE	AMOUNT OF NUCLEIC ACID GIVEN TO MICE WITH THE DIFFERENT PREPARATIONS (AS COMPARED WITH THE CONTENT OF NUCLEIC ACID IN ORIGINAL NPD)
	mg		
27 C-1 Control original NPD	0.01	1/6	1
27C-1C Coagulable protein obtained in first step	0.005	1/8	1/4
27 C-1D (PF) Acid ppt'd protein obtained in second step	0.005	0/6	1/15
27 C-2F (NA) Fraction freed of protein & containing nucleic acid (step 4)	0.05	4/4	30

* See text for the preparation of these fractions.

Autopsies on normal pigs which died from the injection of NPD and on sensitized pigs in which delayed death occurred after these injections revealed the picture of toxemia: abundant exudate in the peritoneal cavity and inflammation of the peritoneum and of the suprarenal glands, which were dark brown in color and hemorrhagic. These symptoms occurred in guinea pigs that died in three hours (or longer) after injection of *H. pertussis* NPD. Sensitized guinea pigs died shortly after injection with NPD, and on autopsy showed the typical picture of anaphylactic shock, with enlarged lungs and greatly extended heart.

The lethal dose of *H. pertussis* NPD for normal guinea pigs is equal to about 2 mg of nitrogen per kilogram of body weight. Some normal guinea pigs died from even smaller doses—0.5 and 0.35 mg of nitrogen. Apparently guinea pigs are much more susceptible to NPD than mice. Sensitized guinea pigs died from 0.15 mg or less of NPD nitrogen.

Another feature is that *H. pertussis* NPD as compared with bacterial toxins (staphylococcus, diphtheria, etc.) kills normal guinea pigs in a rather short period of time, from 3 to 16 hours. It is interesting to notice that, whereas previously described toxic substances isolated from *H. pertussis* filtrates (exotoxin) or from the organism itself (endotoxin) are unstable, the solutions of NPD are very stable and can be Berkefeld-filtered and stored at room temperature for many months without losing their toxicity.

TABLE 3

Toxicity of H. pertussis NPD for normal and sensitized guinea pigs

H. PERTUSSIS PROTEIN INJECTED: MG N PER KG BODY WEIGHT	NORMAL GUINEA PIGS		SENSITIZED GUINEA PIGS	
	Results	Autopsy	Results	Autopsy
8.5	Died overnight	Toxemia		
5.5	Died overnight	Toxemia		
4	Died overnight	Toxemia	Died in 8 minutes	Anaphylactic shock
3	Died overnight	Toxemia	Died in 5 minutes	Anaphylactic shock
3	Died overnight	Toxemia		
3	Died overnight	Toxemia		
3	Died in 6½ hours	Toxemia		
3	Died overnight	Toxemia		
2	Died overnight	Toxemia	Died in 6 minutes	Anaphylactic shock
0.9	Died in 3¼ hours	Toxemia	Died overnight	Toxemia
0.9	Died overnight	Toxemia	Died in 9 minutes	Anaphylactic shock
0.9	Died overnight	Toxemia	Died in 8 minutes	Anaphylactic shock
0.9	Died in 4½ hours	Toxemia	Died in 4 minutes	Anaphylactic shock
0.5	Survived		Died in 4 hours	Toxemia
0.5	Died in 5 hours	Toxemia	Died in 3 hours	Toxemia
0.35	Survived		Died in 3 minutes	Anaphylactic shock
0.35	Died overnight	Toxemia		
0.15			Died in 2 minutes	Anaphylactic shock
0.07			Died in 4 minutes	Anaphylactic shock
Total	17 guinea pigs		12 guinea pigs	

Experiments with the Dale [1] *test on isolated guinea pig uterus.* These experiments, adapted from Burn (1928), were performed on isolated uteri of normal and sensitized virgin guinea pigs. Sensitized pigs were prepared by intra-abdominal injection of *H. pertussis* vaccine two or three months before the experiment. The most satisfactory results were obtained when the uterus was kept in Locke-Ringer's solution with a continuous flow of oxygen. This solution was made according to the formula given in the U. S. Pharmacopoeia.

In the course of this work we tested normal and sensitized guinea pigs with different batches of NPD, the concentration of which was expressed in dilution of nitrogen content. At the completion of each test the vitality of the uterus was tested with histamine dihydrochloride, 1:10 million. The uteri of normal pigs did not respond to NPD diluted 1:1 million, whereas the uteri of highly sensitized pigs contracted on addition of NPD diluted 1:100 million.

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[1] Dale, 1913, 1929; Dale and Kellaway, 1922.

DISCUSSION

The literature contains much information on sensitivity to nucleoproteins and on anaphylactic shock produced by these substances. The earlier observation of Freund (1920*a*, 1920*b*) on the shock produced by the injection of fresh defibrinated blood into animals was later traced by Zipf and Wagenfeld (1930), Zipf (1931), and Barsoum and Gaddum (1935) to the appearance in the blood of adenyl compounds derived from the breaking down of nucleoproteins. Drury (1936) thinks that a combination of histamine and adenyl compounds may be sufficient to account for the whole of the vessel reaction and leucocytosis seen after injury, but he warns that "it would be rash to assume that they are the only substances responsible for the complex response of inflammation."

The significance of nucleoproteins in bacterial allergy has been discussed many times, particularly in connection with sensitivity to tuberculin. The literature on protein in tuberculin cannot be reviewed here. It is necessary, however, to mention the work of Seibert (1940), whose technique we have been using. This author studied the interrelation of nucleoprotein to the other protein of tuberculin in the development of skin sensitivity. Stahl *et al.* (1939) and Huddleson (1943) prepared nucleoprotein from *Brucella* for testing skin allergy in brucellosis. The relation of bacterial allergy to other kinds of hypersensitivity has been reviewed by Rich (1941).

It is interesting to compare the chemical analysis of NPD (nucleoprotein, denatured, prepared from *H. pertussis*) with similar materials prepared from other sources and reported in the literature. For instance, the nitrogen and phosphorus content of several batches of NPD prepared by us were 12 to 15 per cent and 4 to 6 per cent, respectively—the proportion of nitrogen and phosphorus in these preparations being about 3.8:1. Claude and Potter (1943) found 15.58 per cent nitrogen and 3.72 per cent phosphorus in chromatin threads from resting nuclei of leukemic cells. Carter and Hall (1940) found in nucleohistone of calf thymus 16.73 ± 0.2 per cent nitrogen and 4.6 ± 0.1 per cent phosphorus, and Mirsky and Pollister (1943) found 15.5 per cent nitrogen and 3.9 per cent phosphorus in fibrous nucleoprotein of chromatin. Tipson (1945) recently discussed in detail the difficulty of establishing the homogeneity of different nucleoprotein preparations in connection with their chemical composition. Sevag *et al.* (1941) and Lackman *et al.* (1941) isolated very pure streptococcal nucleic acid that contained 16.2 per cent total nitrogen and 9.12 per cent total phosphorus. The sample of nucleic acid that Levene and Bass (1931) obtained from tubercle bacilli, which most nearly approaches desoxyribo-*tetra*-nucleotide, contained 14.19 per cent and 9.04 per cent, respectively, of nitrogen and phosphorus as compared with the theoretical values of 16.76 and 9.89.

We were able to break down our product by boiling it at slightly alkaline reaction followed by precipitation of the remaining protein by acidification.

In this way we could prepare a solution of nucleic acid that reacted negatively to the biuret test and gave a positive reaction for nucleic acid and carbohydrate. This purified nucleic acid did not produce anaphylactic shock in sensitized animals. In this respect our work with *H. pertussis* NPD confirmed the previous findings of other authors insofar as laboratory animals became sensitive to the protein of this material but not to the nucleic acid. Similar findings were reported by Stahl, Pennell, and Huddleson (1939) on nucleoprotein of *Brucella*, and by Seibert (1940) on tubercular proteins.

The allergenic properties of *H. pertussis* organisms apparently represent a complicated mechanism of bacterial allergy. Sabin and Joyner (1938) in very comprehensive experiments demonstrated that injection of a tuberculo-phosphatide accelerated sensitization of guinea pigs to tuberculo-protein. In our case, the removal of lipoids from *H. pertussis* bacilli by extraction with a mixture of alcohol and ether containing 1 per cent hydrochloric acid reduced the sensitizing properties of *H. pertussis* vaccine on laboratory animals. However, our treatment does not destroy entirely the sensitizing properties, and NPD prepared from such extracted organisms can induce some sensitivity in rabbits and guinea pigs, as will be described in the following paper, although the injection of even large doses of denatured *H. pertussis* NPD does not induce anaphylactic sensitivity in mice.

Sensitivity to *H. pertussis* crosses with a few other related gram-negative organisms. However, this condition is not connected with any increase in the susceptibility of mice to injection of proteinic material in general. As compared with normal animals, mice sensitized to *H. pertussis* proteins did not show a noticeable increase in susceptibility to injections of horse serum, rabbit serum, commercial tuberculin, broth used for liquid media, etc.

SUMMARY

Laboratory animals injected with *Hemophilus pertussis* antigens (vaccine or filtrate) develop sensitivity to proteins of these organisms, particularly to nucleoproteins. Fractionation of the nucleoprotein complex revealed that animals sensitized to *Hemophilus pertussis* became highly susceptible to the protein portion of this complex.

The sensitizing properties of *Hemophilus pertussis* antigens were reduced after treatment with a mixture of alcohol and ether containing 1 per cent hydrochloric acid.

Hemophilus pertussis nucleoprotein (NPD) can be used as a shocking fraction for testing sensitivity.

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A STUDY OF SENSITIVITY TO HEMOPHILUS PERTUSSIS IN LABORATORY ANIMALS

III. The Formation of Antibodies and the Development of Sensitivity in Laboratory Animals Injected with Hemophilus pertussis Antigens

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The antigenic response of laboratory animals to injections of *Hemophilus pertussis* exotoxin, vaccine, and nucleoprotein (NPD)[1] was measured. For this purpose we prepared hyperimmune sera in rabbits against each of these antigens.

Antitoxic serum was prepared by repeated injections into rabbits over a long period of time of filtrates of *H. pertussis* culture detoxified with formalin. During such a series of injections the rabbits develop antibodies that neutralize the toxic principle of *H. pertussis* filtrate. This toxin, it should be mentioned, is very unstable and of low titer. Owing to the fact that the filtrates of *H. pertussis* culture contain an abundance of somatic antigens, the serum of rabbits immunized with culture filtrates contains, in addition to antitoxin, high titers of precipitins, agglutinins, and other antibacterial antibodies. However, the integrity of *H. pertussis* toxin as an antigen is supported by the fact that antibacterial serum prepared by immunization of rabbits with *H. pertussis* vaccine does not contain an appreciable amount of antitoxic antibodies.

The origin of the toxic principle in filtrates of *H. pertussis* culture is not clear. Strean and Grant (1940) extracted toxin from the bodies of *H. pertussis* organisms (endotoxin) and prepared immune serum against it in rabbits. Evans (1940) found that this antitoxin neutralized pertussis, parapertussis, and bronchiseptica toxins without differentiation.

We were concerned only with the different effects observed following the injection of toxin-antitoxin mixtures into normal and sensitized mice. *H. pertussis* toxin, 0.1 ml, containing 2 MLD (as determined for normal mice) was incubated for 1 hour at room temperature with different amounts of antitoxin. The mixture in a volume of 0.2 ml was injected intravenously into both normal and sensitized mice. The results of some of our experiments are given in table 1. The amounts of antitoxin used in the mixtures, with the exception of the last one, were sufficient to make these injections harmless for normal mice. In contrast to this, we observed a high incidence of deaths in sensitized mice even after the injection of a mixture containing a large dose of antitoxin. As stated before, in these experiments we used toxin containing 2 MLD, the volume of toxin being not more than 0.1 ml. This volume of toxin did not contain enough allergenic material to kill sensitized mice (see paper I). Apparently, the antitoxin did not protect sensitized mice against toxin as completely as normal animals.

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[1] See paper II in this series.

TABLE 1

Passive protection of normal and sensitized mice against H. pertussis toxin with antitoxic serum

ANTITOXIC SERUM		TOXIN	SENSITIZED MICE	NORMAL MICE
Dilution	Volume of antitoxin injected			
	(ml)			
und.	0.1	2 MLD	3/4*	6/6
1:5	0.02	2 MLD	1/6	4/4
1:10	0.01	2 MLD	6/10	8/8
1:20	0.005	2 MLD	7/12	4/4
1:50	0.002	2 MLD	8/14	6/6
1:100	0.001	2 MLD	0/4	6/6
1:200	0.0005	2 MLD		2/2
1:500	0.0002	2 MLD		0/2

* In all the tables in this paper the total number of mice used is shown in the denominator, the number of survivals in the numerator.

TABLE 2

Precipitin tests of AF and NPD with antibacterial and anti-NPD sera

SERUM 0.1 ML UNDILUTED	ANTIBACTERIAL SERUM 831B		ANTI-NPD SERUM 864A	
Antigen doubling dilutions in volume of 1 ml	*AF 90B106A*	*NPD 29C-2*	*AF 90B106A*	*NPD 29C-2*
und.	++	±	++++	±
1:2	++++	±	++++	±
1:4	++++	±	++++	++
1:8	++++	−	++++	++++
1:16	+++	−	++++	++++
1:32	++	−	++++	++++
1:64	+	−	++++	++
1:128	+	−	+	++
1:256	−	−	+	++
1:512	−	−	−	+

Antibacterial serum was prepared by immunizing rabbits with intravenous injections of *H. pertussis* vaccine (Kendrick *et al.*, 1935–36). Serum was prepared against nucleoprotein by injecting rabbits with alum-precipitated NPD once a week for 4 consecutive weeks, or longer. Table 2 summarizes precipitin tests performed with antibacterial and anti-NPD sera against AF and NPD. In these tests 0.1 ml undiluted serum was added to 1 ml of different dilutions of antigen.

It is apparent from table 2 that anti-NPD serum contains a high titer of antibodies against both nucleoproteins and antigens soluble in filtrates of *H. pertussis* culture, whereas antibacterial serum has only traces of antibodies against nucleoproteins although it possesses a high titer of antibodies against soluble antigens in culture filtrates.

Both sera were also tested for their capacity to agglutinate *H. pertussis* organisms. According to the rapid slide agglutination method, different batches of antibacterial serum showed positive reactions in dilutions up to 1:4,000 to 1:8,000. In contrast to this, different samples of anti-NPD serum possessed very little or no agglutinins for *H. pertussis* organisms.

In continuing this work we investigated the possibility of producing passive transfer of sensitivity in mice and guinea pigs with antitoxic, antibacterial, and anti-NPD sera. Serum was injected intra-abdominally into guinea pigs which were tested 24 hours later by intracardial injection of NPD or vaccine. The average weight of the guinea pigs used was 410 to 420 grams. Table 3 records the results of tests performed with normal guinea pigs and those passively sensitized with anti-NPD serum.

TABLE 3

Passive transfer of sensitivity to H. pertussis nucleoprotein by injection of anti-NPD serum into guinea pigs

AMOUNT OF NPD INJECTED INTRACARDIALLY 24 HR AFTER SERUM—CALCULATED IN MG N OF NPD PER KG BODY WEIGHT OF GUINEA PIG	NORMAL GUINEA PIGS—RECEIVED NO SERUM	GUINEA PIGS INJECTED WITH ANTI-NPD SERUM
mg		
0.24	4/5	0/16

Passively sensitized guinea pigs were challenged with a dose of nucleoprotein containing 0.24 mg nitrogen per kilogram of body weight. One out of five control normal guinea pigs died the day after receiving such a dose, whereas all 16 of the pigs passively sensitized with anti-NPD serum died—most of them after 4 to 5 minutes with symptoms of severe anaphylactic shock. This sensitivity was induced by anti-NPD serum injected intra-abdominally in amounts of 0.25, 0.5, and 1.0 ml.

We were unable to demonstrate passive transfer of sensitivity with *H. pertussis* antitoxic serum, although some batches of antibacterial serum exhibited this effect.

Passive transfer by injection of anti-NPD serum apparently also increased sensitivity of guinea pigs to *H. pertussis* vaccine. In an experiment performed with normal guinea pigs weighing about 415 grams, each was injected intracardially with 1 ml of *H. pertussis* vaccine containing 30 billion organisms per ml. Three pigs, used as controls, which did not receive a preparatory injection of serum, survived injection of the vaccine. The remaining 5 guinea pigs each received 1 ml of anti-NPD serum intra-abdominally, followed 24 hours later by an injection of vaccine. Of these 5 pigs, 4 died with symptoms of anaphylactic shock.

Injection of a sublethal dose of NPD into guinea pigs sensitized by passive transfer leads to desensitization of the animals. A group of normal guinea pigs averaging 404 grams was passively sensitized by intra-abdominal injection of 1 ml of anti-NPD serum. Nine of these pigs were injected intracardially 24 hours later with NPD containing 0.06 mg nitrogen calculated per kilogram of body weight (about one-fourth the lethal dose for passively sensitized guinea pigs). Twenty-four hours after the desensitizing injection the pigs were tested with one lethal dose of NPD, corresponding to 0.24 mg nitrogen per kg body weight, and all 9 survived.

Other guinea pigs, similarly sensitized by passive transfer, were "desensitized" by an intracardial injection of *H. pertussis* broth. (With this injection each pig received 0.09 mg nitrogen calculated per kg of body weight.) The pigs were challenged 24 hours later by injecting NPD in the same dose as was used for the previous group. Three out of four pigs died from anaphylactic shock.

TABLE 4

Desensitization of passively sensitized guinea pigs

DESENSITIZING INJECTION GIVEN 24 HR AFTER PASSIVE TRANSFER	A	B	CONTROL
	Nucleoprotein solution (NPD) 0.06 mg N per kg body weight	*H. pertussis* broth 0 09 mg N per kg body weight	Without desensitizing injection
(Shocking injection given intracardially 48 hr after passive transfer. NPD containing 0.24 mg N per kg body weight)	9/9	1/4	0/7

Seven passively sensitized guinea pigs were saved as controls. These received no desensitizing injections, but were challenged with a shocking dose (0.24 mg nitrogen) of NPD. All died of anaphylactic shock.

From table 4 it can be seen that injections of sublethal doses of NPD have a desensitizing effect on guinea pigs sensitized by passive transfer. The results shown in the middle column are an indication of the specificity of this phenomenon since the injection of broth did not protect 3 out of 4 pigs from anaphylactic shock.

Some successful experiments dealing with passive transfer in mice were performed. The mice were sensitized by intra-abdominal injection of 0.5 ml of rabbit anti-NPD serum, and 24 hours later were challenged with an intravenous injection of NPD solution containing 0.5 mg nitrogen per dose. Mice which were challenged with the same dose of NPD without having received a previous injection of serum served as controls. The results of this test appear in table 5.

This test demonstrated passive transfer of sensitivity in mice that lasted at least 24 hours. In such experiments, sensitized mice died within ½ to 1 hour after the shocking injection. Although we succeeded in producing passive transfer in the foregoing test, it was a very moderate type of sensitivity as compared with that attained by active sensitization (see paper II).

Some evidence was obtained of the desensitizing effect of a minute dose of NPD on such mice moderately sensitized by passive transfer. The fact that the shocking injection was given 48 hours after passive transfer presented some difficulty, since by that time some of the mice were spontaneously desensitized. However, the difference in incidence of deaths occurring after a shocking injection in mice which had received a desensitizing injection and in those which had not received one seems significant enough to mention here. The difference is illustrated by the results of some of our experiments which are summarized in table 6.

So far, our experiments on desensitization of mice actively sensitized by the injection of *H. pertussis* vaccine have given inconsistent results.

TABLE 5

Passive transfer in mice

	MICE PASSIVELY SENSITIZED WITH ANTI-NPD SERUM	CONTROL MICE (NOT INJECTED WITH SERUM)
(Shocking injection of NPD contained 0.5 mg N per dose—injected 24 hr after passive transfer)	3/30	13/16

TABLE 6

Desensitization of passively sensitized mice

	MICE DESENSITIZED I.V. WITH NPD CONTAINING 0.05 MG N PER DOSE	CONTROL MICE WITHOUT DESENSITIZING INJECTION
(Shocking injection 48 hr after passive transfer—NPD containing 0.5 mg N per dose)	24/24	10/24

In pursuing this phase of the work, we also experimented with local passive transfer by injecting guinea pigs intradermally with rabbit anti-NPD serum. Twenty-four hours later the sites of these injections were tested by intradermal injection of a solution of NPD. As a control, several sites on the other side of the same guinea pig were injected with normal rabbit serum and subsequently tested with NPD. After the injection of NPD into the sites sensitized by anti-NPD serum, we observed the almost immediate appearance of a red area, which developed into a wheal within 20 minutes, after which time the redness faded to a slight pink color.

Several experiments are summarized as follows: Guinea pigs weighing about 400 grams received on one side three 0.1-ml intradermal injections of anti-NPD serum diluted 1:20, and on the opposite side three 0.1-ml injections of the same dilution of normal rabbit serum. The next day all six sites were injected with 0.01 ml NPD containing 0.01 mg nitrogen. The sizes of the wheals, measured after 20 minutes, are given in table 7. Each figure represents the average measurement of 36 reactions.

When using strong serum, we were able to sensitize passively local skin sites in guinea pigs by injecting 0.1 ml of anti-NPD serum diluted 1:1,000, and to obtain a wheal by reinjecting with NPD solution containing 0.001 mg nitrogen.

DISCUSSION

Antibodies against nucleoproteins of different bacteria have been discussed in the literature by Lancefield (1925, 1928), Mudd and Lackman (1941), Mudd and Wiener (1942), Smadel *et al.* (1942), and Heidelberger and Kendall (1931). The work we have done with *H. pertussis* is well in line with the findings of Lancefield, i.e., that in streptococci the nucleoprotein is a true antigen which stimulates the development of antibodies in rabbits and produces anaphylactic shock in guinea pigs actively and passively sensitized. Our work coincides in other respects with the observations of Lancefield. Serum produced by immunization with *H. pertussis* NPD similarly contains precipitins but no agglutinins.

TABLE 7

Local passive transfer of sensitivity to guinea pigs

	SITES SENSITIZED WITH NORMAL RABBIT SERUM	SITES SENSITIZED WITH ANTI-NPD SERUM
Average diameter of wheal	8 mm (5–12 mm)	18 mm (9–23 mm)

Several authors have succeeded in performing passive transfer of sensitivity to guinea pigs with rabbit antibacterial serum, and in shocking them with the corresponding carbohydrates. In this respect the investigations of Avery and Tillett (1929) with pneumococci and of Enders (1929) with tubercle bacilli are especially interesting. In our work, passive sensitization permitted us to demonstrate in guinea pigs, and to some extent in mice, desensitization with small doses of NPD.

Recently Mayer and Brousseau (1946) reviewed the subject of anaphylaxis in mice. It was the general conclusion of these authors that "protein shock" in mice is a true anaphylaxis. However, the mechanism of sensitization in these animals and the role of histamine remains unknown. These authors sensitized mice to horse serum by injecting a total dose equivalent to about 60 mg nitrogen, whereas in our experiments a total dose of *H. pertussis* vaccine containing 0.2 mg nitrogen was effective. However, the sensitivity to *H. pertussis* is influenced by the amount of antigen injected. Apparently they did not have any difficulty in desensitizing mice which were hypersensitized to different sera. We were able to desensitize mice only after moderate sensitization to *H. pertussis* nucleoprotein (NPD) after passive transfer.

SUMMARY

The preparation of serum with a high titer of antibodies against nucleoprotein (anti-NPD serum) was accomplished.

Passive transfer of sensitivity in guinea pigs and mice with rabbit anti-NPD serum was demonstrated.

Desensitization of passively sensitized guinea pigs and mice was produced with minute amounts of *Hemophilus pertussis* NPD.

Local passive transfer in guinea pigs was observed.

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THE USE OF SODIUM AZIDE FOR DETERMINING THE FERMENTATIVE ABILITY OF YEAST DEVELOPED UNDER DIFFERENT OXYGEN TENSIONS

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Since the time of Pasteur quantitative differences in the rate of fermentation or respiration have been reported for yeast developed under different oxygen tensions. Fermentative activity in the majority of cases has been based on rates of CO_2 production, although to a limited extent rates of glucose utilization and the rates of alcohol formation have also been employed. It should be emphasized that, in the absence of oxygen, an evaluation of CO_2 produced or of alcohol formed is an index of fermentative activity, whereas the measurement of glucose utilization includes both fermentative and assimilatory processes. In the presence of oxygen, as indicated in studies relating to the Pasteur effect (Lipmann, 1942), fermentative processes tend to be depressed, and, if the medium is suitable, assimilatory processes are enhanced.

As will be shown subsequently, identification of the relative fermentative activity of yeast developed under different oxygen tensions is confounded by cell multiplication. New cells formed after inoculation of the test medium obviously cannot be regarded as representative of cells developed under the conditions characteristic of the inoculum.[2] Moreover, with cells developed under certain conditions the rate of glucose utilization per cell does not remain constant but diminishes with the length of the observation period.

Pickett and Clifton (1941) have indicated that sodium azide in a concentration of 10^{-4} M inhibits both the assimilatory and the respiratory processes of yeast. Winzler (1944) has shown that with washed bakers' yeast in the presence of 10^{-3} M azide, glucose is metabolized quantitatively to CO_2, whereas in the absence of azide, assimilatory processes account for a significant portion of the glucose metabolized. From observations made on the inhibition of phosphate uptake in the presence of azide, Spiegelman, Kamen, and Dunn (1946) have suggested that azide interferes with the generation of high-energy phosphate bonds through the oxidative coupling reaction. If this is true, it follows that in the presence of appropriate concentrations of azide fermentative processes should not effect a net increase in high-energy phosphate bonds. Based largely on this explanation for the action of azide and on the observation that the rate of glucose metabolism under certain circumstances is increased three to four times by the presence of azide, is Brockmann and Stier's proposal (1947*b*) that, in the presence of azide, fermentation is released from partial inhibition resulting from a lack of outlets for accumulated high-energy phosphate bonds. This explanation is somewhat analogous to that proposed by Meyerhof and Junowicz-Kocholaty (1942) in connection with the stimulating action of arsenate on fermentations induced with a cell-free yeast preparation.

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[1] Seagram Research Associate at the Laboratory of Cell Physiology, Indiana University, 1944–1946, on special assignment from Joseph E. Seagram and Sons, Inc., Louisville, Kentucky.

[2] The authors have observed a doubling or trebling of the initial yeast population following inoculation of a glucose-KH_2PO_4 solution with washed cells developed in cotton-plugged flasks. The problem of cell growth is not eliminated by the use of a medium containing no source of available nitrogen.

This paper deals with the rates of glucose utilization in growth medium sparged with tank CO_2 and inoculated with yeast developed under different oxygen tensions: (1) in cotton-plugged flasks, (2) under aeration, and (3) under continuous sparging with tank CO_2 (oxygen content below 0.009 per cent). It will be pointed out that a number of serious difficulties surround the interpretation of such rate data. These difficulties are eliminated when a small amount of azide is added to the growth medium. In addition, consideration will be given to the problem of unit cell population and unit cell weight as a basis for expressions of the rate of glucose utilization and estimations of the fermentative ability of yeast having different oxygen histories.

METHODS AND MATERIALS

Yeast strain. The yeast (distillery type) employed in this investigation was obtained from Joseph E. Seagram and Sons, Inc., Louisville, Kentucky, under the identification, *Saccharomyces cerevisiae*, strain DCL. This strain is included in the yeast collection of the Northern Regional Laboratory, Peoria, Illinois.

Medium. The medium used throughout this investigation contained 10 g glucose, 0.7 g yeast extract (Difco), and 0.5 g KH_2PO_4 per 100 ml. Volumes of 400 to 600 ml were sterilized by heating at 118 to 120 C for 10 to 12 minutes.

Preparation of inoculum. Three types of inocula have been employed in the course of this investigation. For convenience, these will be designated as (1) aerated, (2) initially aerobic, and (3) CO_2-sparged. Each type was started from a common stock culture. This stock culture was carried in tubes of solid medium (the foregoing medium plus 2 g agar per 100 ml). The stock culture was transferred on the first and fifteenth of each month; after incubation for 1 day at 30 C, the culture was held at 3 to 5 C.

In the preparation of aerated inoculum, a tube containing 10 ml of medium was inoculated from the stock culture. After incubation for 1 day at 30 C, one ml of culture was used to inoculate another 10-ml portion of medium. Following 1 day's incubation at 30 C, four ml of this culture were used to inoculate 500 ml of medium in a liter Florence flask. This medium was held under continuous agitation in a 30 C water bath and sparged with 40 ml of sterile air per minute. After 16 to 16.5 hours' incubation, a definite volume of this culture was taken as inoculum for experimental fermentations. After the indicated period, the yeast population in the inoculum culture was ca. 300 million cells per ml while the residual glucose was more than 2 g per 100 ml.

In the preparation of initially aerobic inoculum, the contents of the tube representing the second transfer in liquid medium, as described in the preceding paragraph, were used to inoculate 150 ml of medium in a cotton-stoppered flask. This was held in a 30 C incubator for 1 day before use as experimental inoculum.

Inoculum of the type designated as CO_2-sparged was maintained for more than three transfers in a medium continuously sparged with tank CO_2. The apparatus and operating procedure for the preparation of this type of inoculum have been described by Brockmann and Stier (1947*a*). Apparatus and operating procedures were directed toward the exclusion of air from the culture.

Management of fermentation. All fermentations were conducted in 1-liter all-glass fermentation flasks (Brockmann and Stier, 1947*a*), which were held under continuous agitation in a 30 C water bath. Before inoculation the fermentation flask which contained 500 to 550 ml of medium was sparged with tank CO_2, rate ca. 80 ml per minute, for at least 1 hour. The inoculum before transfer into the fermentation vessel was thoroughly sparged with CO_2. After inoculation, sparging was continued for 10 to 15 minutes. During the course of fermentation the vessel was vented through a mercury seal. The procedure for withdrawing periodic samples during the fermentation period has been described by Brockmann and Stier (1947*a*).

Glucose concentration. The concentration of glucose in the medium was determined by the procedure of Shaffer and Somogyi as modified by Sumner and Somers (1944). Within a minute after withdrawal of a sample, 5 ml were transferred to a volumetric flask containing 2 ml of 5 N H_2SO_4. The acidified sample was held at 3 to 5 C until analytical operations were initiated. Before diluting the sample to a sugar concentration appropriate to the range of the reagent, sufficient 1 N NaOH was added to bring the sample to neutrality (phenolphthalein indicator).

Yeast population. The number of yeast cells in a known dilution of medium was determined by direct count in a Neubauer counting chamber. All well-defined buds which were more than one-fourth the size of the parent cell were counted as separate cells. In view of the indeterminate activity of yeast buds for the metabolism of glucose, the calculated rate of glucose utilization per unit cell population should be regarded, during periods of extensive yeast growth, as an approximation. In the period between withdrawal of the sample and the start of counting operations the samples were acidified and handled as indicated above.

Dry weight of 10^{10} yeast cells. Yeast cells were centrifuged from a volume of medium estimated to contain between 20 and 200 mg of dry yeast substance. After two washings with water the cells were washed into a volumetric flask and diluted to the mark with ethyl alcohol and water in such proportions that the alcohol concentration in the flask attained 65 to 80 per cent. After a thorough mixing, 5 ml of suspension were withdrawn for a yeast count, which was performed in the manner described for the determination of the yeast population of fermentation medium. An aliquot of the cell suspension was transferred from the volumetric flask to a weighed pyrex dish. This in turn was evaporated to apparent dryness over boiling water. The dish containing the cell residue was held at 45 C in a vacuum oven under 27 to 28 inches of vacuum for 20 hours. After removal from the oven, the weight of 10^{10} cells was calculated from the relationship, W/Yv, in which Y is the yeast population of the water-alcohol suspension in terms of 10^{10} cells per ml and W is the weight in grams for the dry yeast residue from v ml of suspension.

RESULTS

Glucose utilization for each inoculum type. The medium, previously sparged with CO_2, was inoculated with yeast developed under the aerated, initially aerobic, and CO_2-sparged procedures. According to the observations for this experiment which are summarized in figure 1, glucose utilizaton and yeast growth for aerated and initially aerobic inocula are quite comparable. Note that, in both cases, the rate of glucose utilization per unit population decreased with time. The medium inoculated with yeast developed according to the CO_2-sparged procedure showed definitely less cell multiplication and a considerably lower rate of glucose utilization per cell. The glucose utilization rate per population unit, however, remained constant throughout the observation period.

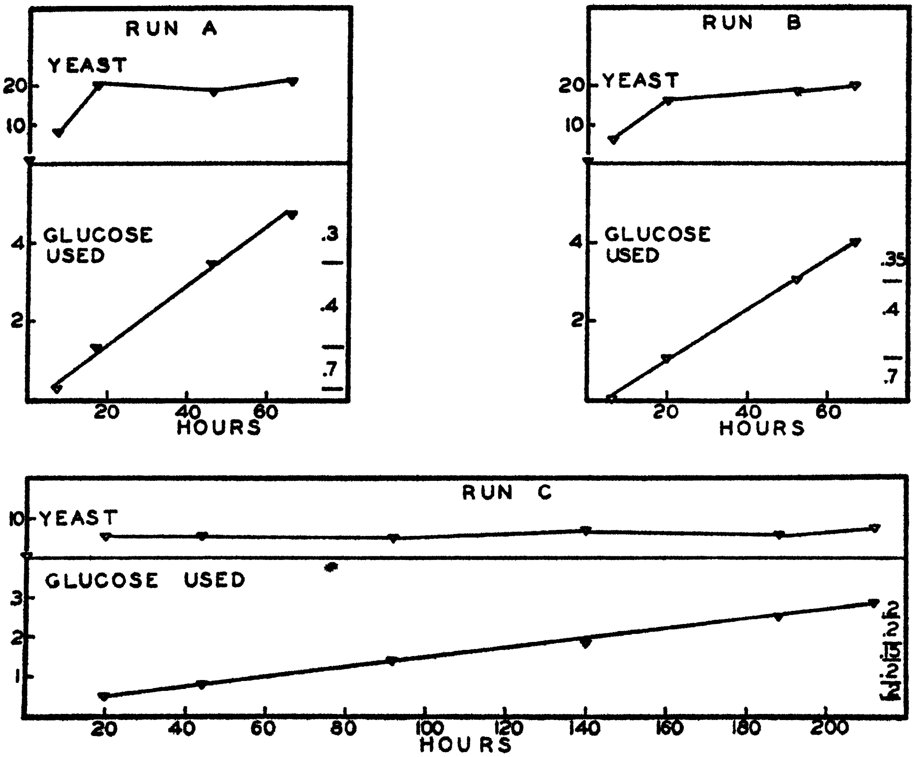

FIG. 1. CO_2-SPARGED MEDIUM INOCULATED WITH CELLS DEVELOPED UNDER DIFFERENT OXYGEN TENSIONS

Run A, inoculum aerated; Run B, inoculum initially aerobic; Run C, inoculum CO_2-sparged.

Yeast as 10^6 cells per ml; glucose used as g per 100 ml. Numbers on glucose-used curve refer to glucose used as g per hr per 10^{10} cells for indicated interval.

Glucose utilization for each inoculum type in medium containing azide, 0.002 M. Cells developed by each procedure for the preparation of inocula were concentrated in a small volume of their culture medium, sparged with CO_2, and introduced into CO_2-sparged medium, which contained sodium azide at a concentration of 0.002 M (after the addition of inoculum). Data for duplicate runs with each inoculum type are summarized graphically in figure 2. In no case is there evidence of yeast growth. In contrast with the preceding experiment, the cells developed under CO_2 had definitely the highest rate of glucose utilization, whereas the cells developed under aeration had the lowest. Cells prepared according to

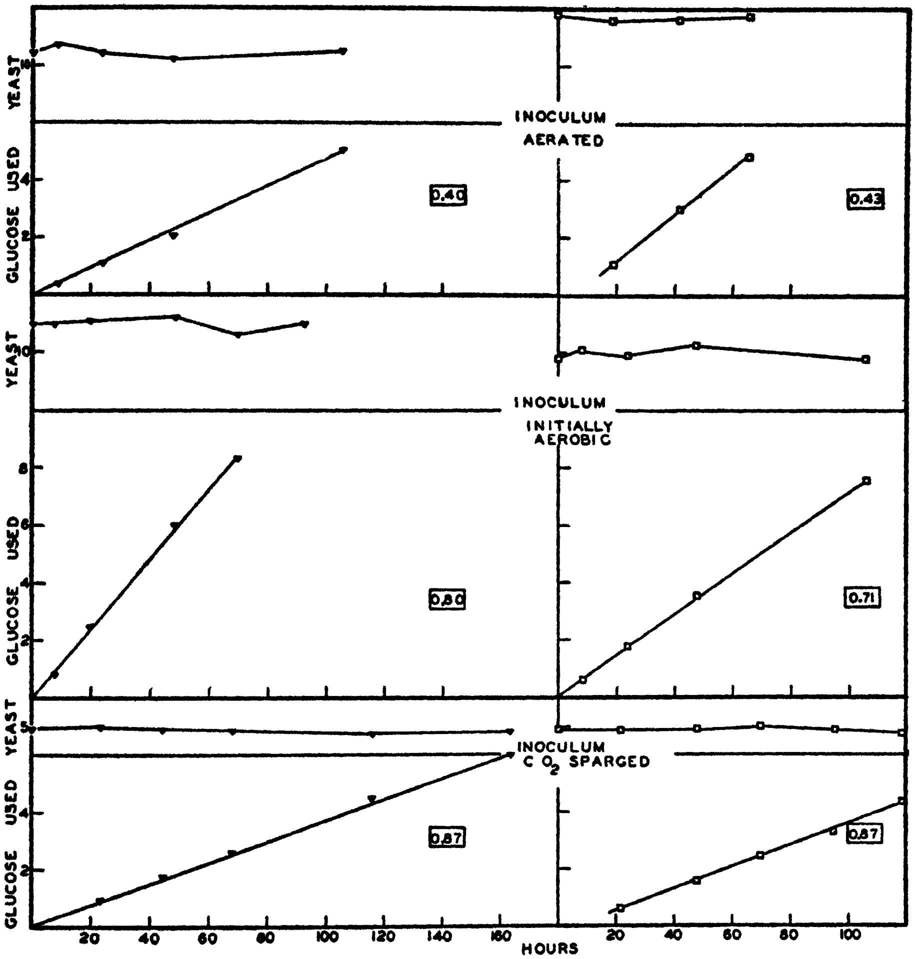

FIG. 2. CO_2-SPARGED MEDIUM CONTAINING 0.002 M AZIDE INOCULATED WITH CELLS DEVELOPED UNDER DIFFERENT OXYGEN TENSIONS

Yeast as 10^6 cells per ml; glucose used as g per 100 ml. Framed numbers on glucose-used curve indicate g glucose used per hr per 10^{10} cells for period of linear relationship.

the initially aerobic procedure were intermediate, somewhat closer to the CO_2-sparged cells.

Cell population versus cell weight as a basis for expressions of fermentation rate. In table 1 are summarized data on the dry weight of 10^{10} cells representing each type of inoculum. Cells developed according to the CO_2-sparged procedure are definitely the heaviest (10^{10} cells = 0.78 g); initially aerobic cells are intermediate (10^{10} cells = 0.38 g); and aerated cells are the lightest (10^{10} cells = 0.27 g). In the course of fermentation in CO_2-sparged medium, cells developed according to the CO_2-sparged procedure gradually lose weight: at 21 hours 10^{10} cells = 0.59 g, at 210 hours 10^{10} cells = 0.46 g. In spite of this weight loss the rate of glucose utilization per cell remains relatively constant throughout the observation period. Aerated cells tend to gain weight in the course of fermentation, whereas initially aerobic cells appear to remain at approximately inoculation weight. However, as is shown in figure 1, both of the latter two cell types have a decreased rate of glucose utilization as fermentation progresses.

TABLE 1

Dry weight of 10^{10} cells representing different inocula types

INOCULA TYPE	WEIGHT 10^{10} CELLS, GRAMS*
CO_2-sparged	0.80
	0.79
	0.74
	0.78
Initially aerobic	0.42
	0.39
	0.34
Aerated	0.28
	0.27
	0.25

For age of cells, see subsection covering preparation of inocula.

* Each value represents an independent run.

TABLE 2

Comparison of rates of glucose utilization on a population and on a weight basis

CELL TYPE	GLUCOSE USED	
	g/hr/10^{10} cells	g/hr/g dry weight
CO_2-sparged	0.87	1.1
Initially aerobic	0.80	2.1
	0.71	1.9
Aerated	0.40	1.5
	0.43	1.6

On the other hand, during fermentation in azide-containing medium, the weight of each cell type tends to remain constant throughout the observation period. After fermentation periods of more than 100 hours in 0.002 M azide, 10^{10} cells from CO_2-sparged, initially aerobic, and aerated inocula were found to weigh 0.77, 0.39, and 0.27 g, respectively. The constancy of cell weight throughout the course of fermentation in 0.002 M azide medium greatly simplifies the calculation of fermentation rate from a unit population to a unit weight basis. Table 2 shows rates of glucose utilization per hour per 10^{10} cells and per g of dry yeast for the observations summarized in figure 2. The data of table 2 illustrate marked lack of parallelism for rates calculated on the basis of cell number and cell weight. Some implications of these observations will be treated in the discussion.

DISCUSSION

On the basis of "classical" theory, the data of figure 1 would be interpreted as indicating that yeast developed under continuous sparging with tank CO_2 is deficient in the enzymatic equipment for the metabolism of glucose. The yeast populations of medium inoculated from initially aerobic and aerated cultures would be regarded as possessing enzyme systems of comparable activity at corresponding periods of fermentation; however, the duration of fermentation would be regarded as a very significant factor in defining the activity of the glucose-utilizing enzyme systems.

From the concept proposed by Brockmann and Stier (1947*b*), it appears that the rate of glucose utilization in CO_2-sparged medium is not a valid index of the fermentative potential of the cell, since glucose utilization is apparently inhibited because of an overaccumulation of high-energy phosphate bonds. If 0.002 M azide releases the cell from the partial inhibition resulting from engorgement of the phosphate-transmitting system with high-energy phosphate bonds generated through fermentative processes, the observed rates of glucose utilization in the presence of azide should represent an adequate index of the cell's fermentative ability. By this method of testing, it appears that yeast developed through several successive transfers in CO_2-sparged medium possesses significantly greater potential for fermentation than cells grown under aeration, and slightly greater potential than cells developed according to the initially aerobic process. From this concept, the fermentation rate in 0.002 M azide is regarded as the maximum attainable by the cell under the environmental conditions employed. Note, however, that aerated cells in the early stages of fermentation in the absence of azide (figure 1) appear to utilize glucose at a rate definitely above that shown for azide-containing medium. It must be recalled that, in the absence of azide, glucose utilization represents both assimilation and fermentation. Furthermore, as the result of multiplication, the yeast population, except at the start of the experiment, does not represent cells developed under aeration.

A number of investigators have assigned a prominent role to the accumulation of alcohol in the depression of the rate of fermentation. As an interesting side-light, the alcohol yield for fermentations conducted in the presence of azide amounted to 94 to 95 per cent of the theoretical based on the glucose used. The fact that fermentation rates remained constant throughout the observation periods is suggestive that low concentrations (up to 4 g per 100 ml) of alcohol do not have a significant effect on the rate of glucose utilization.

Observations made on the weight of dry cells from the three types of yeast inocula clearly illustrate that relative activity based on a unit number of cells may be widely divergent from relative activity based on cell weight. The general aspects of the problem of a suitable basis for indicating fermentative or respiratory activity is beyond the scope of this paper (for citations see Tobias, 1943). However, from the standpoint of the experiments here presented, the use of a unit cell population appears to be a more rational reference standard than cell weight. In all observations based on cell number, data for initially aerobic inocula fall within the range defined by aerated and CO_2-sparged inocula. Moreover, as seen in fermentations inoculated with yeast developed under CO_2 sparging, a significant loss in weight is accompanied by no alteration in the rate of glucose utilization per cell. Accumulations of fat and glycogen, aside from contributing to the weight of the cell, should not add to the mass of fermentation enzymes. The uncritical application of cell weight as employed for the evaluation of Q_{O_2}, $Q^{N}_{CO_2}$, and $Q^{O_2}_{CO_2}$ may result in erroneous interpretations in the relative activity of yeast developed under different environmental conditions.

SUMMARY

If the rate of yeast fermentation is limited under most circumstances by an overaccumulation of high-energy phosphate bonds, conventional procedures are inadequate for evaluating the fermentative ability of yeast. If azide interferes with the generation of high-energy phosphate bonds, the rate of glucose utilization in the presence of azide should represent a suitable index to the fermentative potential of the yeast cell.

On the basis of rates of glucose utilization in CO_2-sparged medium containing azide, yeast cells developed under continuous CO_2 sparging have about twice the activity of cells developed under aeration and about 110 to 125 per cent the activity of cells developed in cotton-plugged flasks.

For yeast developed under different oxygen tensions the rate of glucose utilization per unit time per unit cell population appears to be a more adequate expression of activity than a corresponding rate based on unit cell weight.

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STUDIES ON CELLULOSE FERMENTATION

III. THE CULTURE AND ISOLATION OF CELLULOSE-DECOMPOSING BACTERIA FROM THE RUMEN OF CATTLE[1]

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There seems little doubt that cellulose-digesting microorganisms are extremely important in the digestion of plant materials in cattle and related forms (von Tappeiner, 1884). However, attempts to grow them and to study them under controlled conditions have been almost uniformly unsuccessful. Certain of the rumen protozoa have been shown to digest cellulose (Hungate, 1942, 1943). But their removal from the rumen does not impair cellulose digestion (Becker, Schulz, and Emmerson, 1929), and it must be concluded that other organisms also exercise this function. It has been assumed on the basis of microscopic examination (Henneberg, 1922; Baker, 1942) that these other cellulose-digesters are bacteria.

The present study was undertaken to test for cellulose-digesting bacteria in the rumen by cultural methods. The aim of the experiments has been to culture the cellulose bacteria, isolate them, and to estimate the numbers in which they occur. The latter was deemed essential in order to eliminate those cellulose-digesting species entering the rumen with the food but not participating significantly in the digestion of the cellulose (Ankersmit, 1905).

Belief that the bacteria in the rumen could be grown *in vitro* arose from observations of the flask cultures of the cellulose-digesting rumen protozoa. These cultures were maintained for many months with no additions except dried grass and cellulose. The gas produced in the flasks was found to be chiefly methane and carbon dioxide, the same as the rumen gases. This suggested that the microbial processes in the flask cultures were similar to those in the rumen. It seemed probable that cellulose-digesting bacteria similar to those in the rumen were present in the flask cultures, and the fact that they had grown for an extended period outside their host encouraged the attempt at their isolation.

DEVELOPMENT OF A CULTURE MEDIUM

Agar shake cultures with serial dilutions were adopted as the means of estimating the number of cellulose bacteria. The inorganic medium initially used was the same as that employed for growth of the protozoa (Hungate, 1942). An aqueous extract of dried grass (sterilized by filtration through a Seitz filter) and a sterile filtrate of an active culture of the protozoa were added

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[1] The initial portions of this investigation were given financial support by the Research Institute and the Clayton Biochemical Institute of The University of Texas. The later work has been aided by a grant from The Society of the Sigma Xi.

to the cellulose and agar in the inorganic salt solution. Nitrogen containing 5 per cent carbon dioxide was bubbled through the tubes of melted agar before and after inoculation. The gas was first passed through chromous oxygen absorbent to remove traces of oxygen. Two drops of 1 per cent $Na_2S \cdot 9H_2O$ were also added to each tube just before inoculation in order to absorb any traces of oxygen. The tubes were cooled rapidly under running cold water to give an even dispersion of the cellulose in the agar, and were then incubated at 37 C.

These initial shake tubes failed to show cellulose decomposition, in contrast to the flask cultures of the protozoa. However, the tubes contained a greater concentration of cellulose than did the liquid cultures. This was necessary in order to give sufficient white opacity to permit detection of cellulose digestion. It seemed possible that with this higher concentration of cellulose the low buffering capacity of the medium might not permit sufficient growth of the bacteria to digest a visible amount of cellulose. Accordingly, an inorganic solution containing more phosphate was substituted. This medium supported development of cellulose-digesting bacteria, as evidenced by the appearance of clear spots in some of the tubes.

These clear spots were transferred to new dilution series of similar composition in an attempt to eliminate noncellulose bacteria. As a rule, a clear spot was also inoculated into a parallel series containing a different medium in order to gain information on the essential cultural factors. Thus, in some series the grass extract was omitted, in others the filtrate of the protozoa culture. Some included yeast extract, others a mixture of B vitamins.

The results obtained by these various procedures were extremely conflicting. In one transfer there would appear to be a definite advantage in using protozoan culture filtrate. When the experiment was repeated, quite different results might appear. None of the media tested was found to give reliable growth.

By inoculating several parallel series at each transfer it was possible to subculture 11 successive times, but the twelfth inoculation failed to give further growth. A certain amount of purification of the cellulose decomposer occurred during this time, and when the culture was finally lost there appeared to be only two organisms present. One of these was a small spiral form. The other was a coccus which often was joined in chains, especially in young cultures. When a colony containing these two forms was diluted in a shake series containing glucose, diffuse colonies developed that on microscopic examination were found to contain the spiral. It was inoculated into a cellulose series, but it failed to show cellulose digestion. This suggested that it was not the cellulose decomposer. However, since the culture method was relatively uncertain, another means of ascertaining the nature of the cellulose digester was also employed.

A bit of the cleared agar was diluted in a glucose series, and an approximately equal amount of the uncleared, cellulose-containing agar from an adjacent region was diluted in a similar series. Growth of colonies of the spiral organism took place in both series, and the number was of the same order of magnitude. This indicated that it was not associated primarily with areas showing cellulose digestion.

The morphology of the spiral is shown in figure 1. This organism has been repeatedly encountered in cellulose cultures inoculated with rumen contents. It has also been observed in direct films of rumen contents. It appears to be a usual inhabitant of the rumen.

An ability of the spiral cells to migrate rather rapidly through glucose agar was noted. Since it was necessary to incubate the cellulose cultures for 10 days before clear spots could be detected, there was ample time for it to move throughout the tube. This explains its frequent occurrence as a contaminant in the cellulose cultures. A number of repetitions of this attempt at isolation led to approximately the same results. None of the media employed gave any consistent indication of superiority. Some important variables were causing erratic results. It seemed possible that the noncellulose bacteria constituted one of the important variables and that fluctuations in their numbers and kinds might be of importance.

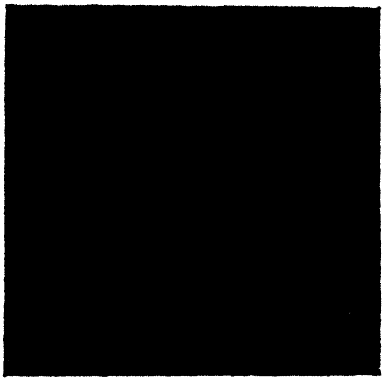

Fig. 1. Spiral Organism from a Cellobiose Agar Culture. Carbol Fuchsin. Magnification about 1,000 ×

The presence of contaminants is usually considered favorable to the growth of cellulose-digesting bacteria. Many investigators have reported failure to obtain cellulose digestion with a pure culture, whereas mixed cultures were active. The success in using mixtures has usually been interpreted as indicating that the accompanying forms in some way aid the cellulose digestion. In the present experiments, however, the culture filtrate should have provided the helpful action of any "synergistic" forms, yet no consistent improvement through its use could be demonstrated. This finally led to the consideration of a different view of the influence of accompanying bacteria. It seemed possible that at least some of them exerted a deleterious effect on the cellulose decomposers and that failure to obtain growth in subcultures was due to overgrowth by other bacteria.

Following this line of reasoning, the culture method was modified in four ways.

(1) Much more careful attempts were made to avoid transferring contaminating forms. (2) The percentage of agar was increased to 2 per cent. This decreased the movement of bacteria in the medium but did not stop it entirely. (3) The inorganic medium was modified to include carbonic-acid bicarbonate as the principal buffer system. The high carbon dioxide content (70 to 75 per cent) of rumen gas coupled with the neutral reaction of the rumen contents indicates that considerable quantities of bicarbonate are present in the rumen. The saliva is the source of this salt. It seemed possible that bicarbonate would be a more natural constituent of the inorganic medium than was the phosphate. Carbon dioxide freed of oxygen was used to displace oxygen and to provide a suitable pH. (4) In order to give more rapid development of the cellulose decomposers, some liquid from the rumen was included in the medium.

For the earlier experiments rumen fluid was obtained at the abbatoir and transported to the laboratory with as little exposure to the air as possible. The samples were obtained by slitting the rumen within 10 minutes after death of the animal, inserting a tubular screen (12 meshes to the inch) into the rumen contents, and drawing into a pipette the liquid and particles which penetrated the screen. For later experiments the liquid was removed by a similar technique from a cow with a rumen fistula.[2]

The liquid rumen contents were immediately boiled, filtered through cotton, and then stored in the refrigerator under an atmosphere of carbon dioxide. In the preparation of the culture medium the agar was dissolved in 4 parts of the inorganic medium. Three parts of cellulose suspension were added and, after boiling, 3 parts of the rumen liquid were added, and the mixture was again boiled. Sodium thioglycolate (0.05 per cent) was added and the medium immediately sterilized at 15 pounds for 15 minutes. On removal from the autoclave the medium was cooled to about 50 C. A solution of sodium bicarbonate (sterilized by filtration) was then added (0.5 per cent final concentration), and the medium was held at 46 C until inoculated.

The composition of the mineral medium before being mixed with the other ingredients was, in percentages: $NaCl$—0.09; $(NH_4)_2SO_4$—0.03; K_2HPO_4—0.05; KH_2PO_4—0.03; $CaCl_2$—0.015; $MgSO_4$—0.015; in tap water.

The cellulose was prepared by treating absorbent cotton with concentrated hydrochloric acid, which caused it to break up into small particles. If the concentrated reagent caused browning, it was diluted with a little water. After 48 hours or more the cellulose was filtered off, washed with tap water, and air-dried. Before use it was suspended in tap water in a concentration of 5 per cent and ground for 72 hours in a pebble mill.

ISOLATION OF THE BACTERIA

Rumen contents were inoculated into the new medium with serial dilutions. Growth of cellulose-decomposing bacteria, as evidenced by the appearance of clear spots, occurred much more rapidly than in any previous series. Cellulose decomposition was evident in the tubes of lower dilution after 2 days, and within a week clear spots 2 mm in diameter were present in some of the higher dilutions. These spots were present not only in the solid agar in the bottom of the tube, but were also in the thin agar lining the upper, gas-filled portion. This was one of the most important results of the new method because it made it possible to transfer only that agar in which the cellulose had been digested, and thus to reduce to a minimum the number of contaminants carried in the inoculum. It was also possible to obtain pure cultures using 1 per cent agar. This concentration was used in most of the later work because it gave more rapid development of the colonies.

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[2] The author is much indebted to Dr. R. W. Dougherty of the College of Veterinary Medicine for making two of these animals available and for aid in withdrawing the samples of rumen fluid.

Colonies were subcultured in cellulose dilution series using a colony in a high-dilution tube for transfer. As soon as the culture appeared to consist of only one kind of colony, it was also subcultured in a parallel glucose or cellobiose agar dilution series. The sugars were sterilized by filtration and added to the medium (0.1 per cent final concentration) after the heat sterilization. The composition of the sugar media was the same as that of the cellulose agar except that tap water and the sugar replaced the cellulose suspension.

If growth occurred in the sugar series, a colony in the highest dilution was subcultured again in a sugar series. Each series was examined for uniformity of the colonies. From a high dilution of this second series, a colony was picked to cellulose. If rapid and typical cellulose digestion occurred and there were no indications of any contaminants, it was concluded that a pure culture of the cellulose-digesting bacterium had been isolated. Four pure cultures were obtained in this way.

These cultures were purified relatively easily as soon as the technique of isolation had been worked out. However, other cellulose-digesting colonies occurring in agar dilution series inoculated with rumen contents have not been grown so successfully. Several of them have shown sporadic growth through a few transfers, but then have failed entirely. Many modifications of the culture medium have been tested in an attempt to obtain consistent growth of all cultures, but as yet no completely satisfactory method has been found. Improvement has sometimes been noted when 2 per cent horse serum was added to the medium just before it was inoculated. It has also been found helpful to carry some strains in a liquid rather than in an agar culture.

A total of 6 different strains of rumen cellulose bacteria have been obtained in pure culture, as judged by successful growth in cellulose after passage through 2 sugar dilution series. Three other cultures were apparently pure but were not grown in sugar.

DESCRIPTION OF ISOLATED STRAINS

In the pure cultures, and in the cellulose-decomposing colonies which have been examined but not isolated, there have been observed two morphological types, a coccus and a rod.

The coccus form was first encountered in the series inoculated from the protozoa cultures, but was not obtained pure from them. It has also been frequently observed in dilution series inoculated from the rumen. Four strains have been purified. The first (strain C) was obtained from the rumen of an animal slaughtered at Austin, Texas. It was transferred through 7 subcultures in cellulose and at the end of that time appeared to be pure, but its failure to grow in glucose prevented the application of the rigorous purity test. No bacteria developed in the glucose agar cultures. On the eighth transfer this strain failed to give further development.

Coccus strain A was obtained from a cellulose agar series inoculated with the rumen contents of a steer killed at Moscow, Idaho. The strain was carried through 4 cellulose agar series. It was then inoculated into a glucose series and showed the development of white, slightly opaque, lens-shaped colonies. One of these in a high dilution was inoculated into another glucose series and from this back into cellulose, in which the organism readily grew.

The other two strains of cocci were obtained from a cow with a rumen fistula. One of them (strain H) occurred initially as an isolated colony in the sixth dilution tube of a cellulose series inoculated with rumen contents. It was subcultured several times in cellulose agar, and appeared to be pure, but would not grow in glucose. The other (strain M) developed in the fifth dilution of a liquid culture series containing cellulose. It was subcultured and purified by repeated transfers in cellulose agar. It failed to grow in glucose but did grow in cellobiose and was pure-cultured. This strain has given more rapid digestion in cellulose agar tubes than has any other strain of bacteria isolated from the rumen, clear spots appearing within 2 days after inoculation.

In all of the strains of cocci the cells average about 1 μ in diameter, though there is much variation from one culture to another, and even within a single colony. Strain C showed a considerable tendency to form chains, as illustrated in figure 2. In some cultures of this strain a definite capsule was present. Strain A showed practically no chain formation in agar (figure 3) but in liquid cultures some loose filaments of from 4 to 8 cells were formed.

The coccus strains usually exhibit a gram-negative reaction. Films of strain A occasionally contain some gram-positive cells. Strains A and M have been observed to include cells containing gram-positive granules though most of the cell was negative. The negative reaction does not appear to be due to the age of the cells since young colonies (2 days old) of strain M have been stained *in situ* in the agar and the peripheral young cells were completely gram-negative.

Young colonies of the cocci give the iodophilic reaction which has been used by Henneberg (1922) and Baker (1942) to identify cellulose bacteria in the rumen by microscopic examination. The staining by the iodine is interpreted as due to glycogenlike material within the cells. Older cells do not exhibit this reaction, as may be seen from the photomicrographs of young colonies (2 days) of strain M in cellulose agar (figure 4). Only the peripheral portions of the colony containing the younger cells give the dark color with iodine.

At various times the coccus strains A, C, and M were inoculated into cellulose agar containing no rumen contents but with yeast extract (Difco) in concentrations ranging from 0.1 to 0.5 per cent. Growth was about the same as in the cultures to which rumen contents were added. The colonies of strains A and C in yeast extract were distinguished by the production of a yellow pigment which diffused out from the colony for a short distance. A similar production of pigment has not been observed in rumen cellulose tubes. The colonies of strain M did not form pigment even when grown in yeast extract.

Fig. 2

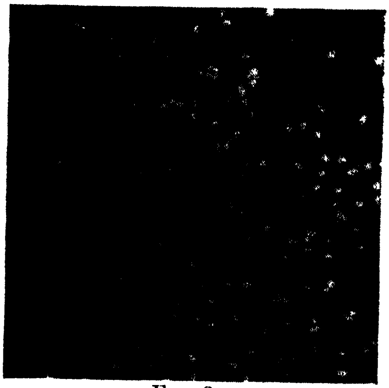

Fig 3

Fig 2 Streptococcus, Strain C, from a Cellulose Agar Culture Nigrosin. Each Small Micrometer Division Equals 0.8 Microns

Fig. 3 Streptococcus, Strain A, from a Glucose Agar Culture Nigrosin Magnification Same as for Figure 2

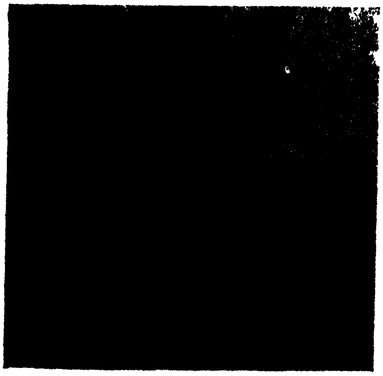

Fig. 4

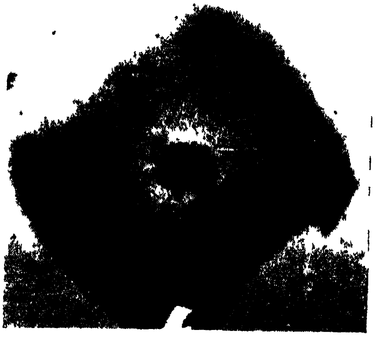

Fig. 5

Fig. 4. Streptococcus, Strain M, 48 hr Colony in Cellulose Agar Iodine. Magnification about 20 X

Fig. 5. Streptococcus, Strain A, Old Colony in Cellulose Agar. Magnification about 6 X

The colony growth of the coccus strains in cellulose agar is quite characteristic. There is no sharp line of demarcation between undigested and digested cellulose such as is observed in *Clostridium cellobioparus* (Hungate, 1944) or *Micromonospora propionici* (Hungate, 1946). The appearance (figure 5) suggests that the cocci are not as effective digesters of the more resistant cellulose. Very young colonies may show relatively little digestion (figure 6).

Reducing materials have been demonstrated in old cultures of strains A and C. The fact that a clearing of cellulose occurs at some distance from the colony (figure 5) shows that an extracellular enzyme is formed. In old cultures it presumably continues to act and produce sugar after the culture is too acid to permit growth of the cells.

No two of the coccus strains that have been isolated have resembled each other in every particular. They have differed in the rate of cellulose digestion, growth in yeast extract, tendency to form chains and capsules, gram reaction, and in pigment production in yeast. However, it seems probable that they are all fairly closely related The morphology suggests that they belong to the genus *Streptococcus*. But their predominantly negative gram reaction is not consistent with this assignment, and for the present it seems preferable to delay naming the organisms. It is possible that the forms described as *Micrococcus ruminantium* and *Streptococcus jodophilus* on the basis of microscopic examination by Henneberg (1922) are the same as the cellulose-decomposing cocci isolated in the present investigation.

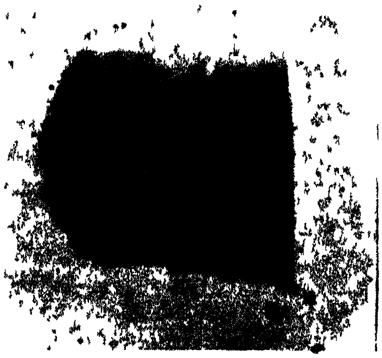

FIG 6

FIG 7

FIG. 6 STREPTOCOCCUS, STRAIN A, YOUNG COLONY IN CELLULOSE AGAR MAGNIFICATION ABOUT 6 ×

FIG 7 ROD FORM, STRAIN A, FROM 4 DAY COLONY IN CELLULOSE AGAR CARBOL FUCHSIN MAGNIFICATION ABOUT 1,000 ×

Six strains of rod-shaped bacteria capable of decomposing cellulose have been obtained from the rumen. Five of them have been obtained in pure culture (A, F, H, R, and S). Another (strain D) was lost before it could be carried through glucose series. All these strains have exhibited several features in common. In fresh mounts the cells are practically invisible, and it is necessary to stain in order to see them. They stain readily with carbol fuchsin but not with methylene blue.[4] Young colonies in cellulose agar consist of small rods 1 μ by 0.3 to 0.4 μ (figure 7). At a later stage the rods almost entirely disappear, and instead are found minute and indistinct spheres of variable size.

The rods disintegrate very quickly after a colony has developed, and subcultures have not been successful when the rods were no longer present. This suggests that the minute indistinct spheres are degeneration products. The rod strains have been uniformly gram-negative. The cells even in young cultures do not stain with iodine except for a slight yellow color when the entire colony is stained. No brown reaction suggesting glycogen has ever been observed. This shows that iodophily alone is not adequate as an index of cellulose-digesting ability by rumen bacteria.

The rods grow readily when glucose or cellobiose is substituted for the cellulose in the medium. When growing on either of these sugars, however, the morphology is quite different from that exhibited with cellulose as the substrate. There is a much greater variation in size and most of the cells are much larger (figure 8) than when grown on cellulose. The large cells stain unevenly, with granules within the cell showing affinity for the dye, whereas the main portions of the cell are barely visible. Old colonies in a sugar medium consist of an amorphous mass of material in which numerous small spheres of variable size predominate.

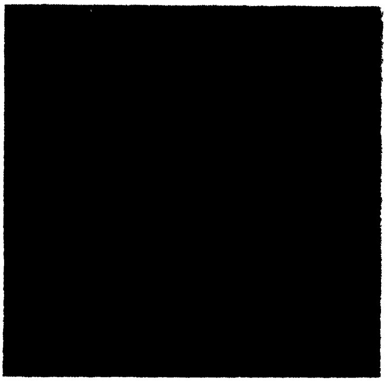

Fig. 8

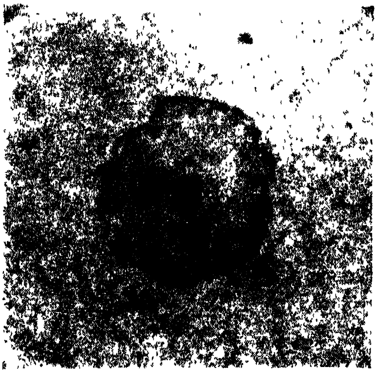

Fig. 9

Fig. 8. Rod Form, Strain F, from 3-Day Colony in Glucose Agar Carbol Fuchsin Magnification about 1,000 ×

Fig. 9. Rod Form, Strain R, from 3 Day Colony in Cellulose Agar. Carbol Fuchsin. Magnification about 40 ×

The rod-shaped cellulose decomposers have exhibited a type of colony growth in cellulose agar which has not been encountered in any other cellulose anaerobes. The first evidence of growth in cellulose agar is the development of a clear space in which the cellulose is completely digested. Within this space no colony can usually be detected, although with strain R a disc-shaped colony may be present in the center of the clear space. By removing a bit of the thin agar containing a young colony and staining the whole colony *in situ* with carbol fuchsin, the active bacteria can be seen as a thin ring in the outermost part of the clear spot immediately adjacent to the cellulose (figure 9). The inner part of the clear area contains few rod-shaped cells, though the indistinct spheres previously mentioned are numerous. The rod form apparently migrates through the agar, and the cells in the center of the colony either become moribund or migrate to the periphery.

There is a sharp line of demarcation between digested and undigested cellulose around colonies of the rod. The location of the cells indicates that relatively close contact with the cellulose is necessary for its digestion, a situation reminiscent of that observed in *Sporocytophaga* (Stanier, 1940). Correlated with this behavior, no reducing materials have been found to accumulate in old cultures of these strains. The several strains of rods have been found to differ slightly from each other in the ease with which they can be cultured and in their rate of growth. However, they all appear to be strains of the same species.

In addition to the strains of cellulose decomposers from the rumen which have been isolated and carried in culture, numerous colonies developing in initial series inoculated with rumen contents have been examined microscopically. In all of these, the cellulose decomposer has seemed to be a rod or a coccus similar microscopically to the strains which have been isolated.

The macroscopic appearance of the thin cellulose agar in which a cellulose-digesting colony from the rumen is growing is sufficient to determine whether the coccus or the rod is concerned. The boundary of cellulose digestion around a colony of the rod is always sharp and distinct, whereas around the coccus it is indefinite. This has made it easy to determine which organism predominated in the quantitative dilution series inoculated with rumen contents.

THE NUMBER OF CELLULOSE BACTERIA AS DETERMINED BY CULTURE METHODS

It has always been possible to demonstrate cellulose-decomposing bacteria in cellulose dilution series inoculated with rumen contents. The numbers per ml of rumen fluid in different animals as found during the initial studies were as follows: A—480,000; B—1,300,000; C—210,000; D—40,000,000; E—18,500; F—13,000,000; G—1,500,000; H—1,200,000,000; and I—780,000.

In all of these original series, except D and H, the cellulose bacteria caused clear spots which gradually increased in size. Few additional colonies developed after further incubation. In the D series, colonies of this sort appeared, and after 13 days one of them in tube 4 was transferred to a new series. The air in tube 4 was displaced with carbon dioxide, and the tube was replaced in the incubator. Seven days later it was noticed that numerous small clear spots were present in the thin agar lining the upper portion of the tube. The volume of this agar was estimated by weighing it, and from the number of colonies present it was calculated that the original inoculum contained 40,000,000 cellulose bacteria per ml.

Previous experience with *Clostridium cellobioparus* had shown that clear spots and streaks in the thin layer of agar might occur if the tube had been laid in a horizontal position. This would allow the liquid which had collected on the surface of the solid agar to spread over the thin region and scatter any bacteria present. Although no indications of this were observed in tube 4 of the D series, it was thought that a mishap of some similar nature might have occurred. However, the same phenomenon was encountered even more strikingly with the series from cow H, and it was concluded that also in the D series the clear spots which appeared after prolonged incubation represented cellulose bacteria which had been present in the original inoculum.

The series inoculated with rumen contents from animal H was incubated for 6 days, at which time clear spots and gas were present in the first three tubes. The colonies were similar in general appearance and behavior to those previously encountered. During the subsequent few days of incubation, colonies appeared in tubes 4 and 5, only one being present in the latter. When the tubes were examined 17 days after inoculation, however, the thin agar in the upper portion of tube 5 showed 152 clear spots. This tube had not been opened and had been incubated in an upright position during the entire period. Furthermore, the single precocious colony was near the bottom of the tube in such a position that there was little chance for bacteria to escape from it into the thin agar layer at the top. It was concluded that the numerous colonies in the thin agar represented cellulose bacteria in the inoculum. In both animals D and H the colonies appearing in such numbers contained only the rod. These initial series were inoculated from animals killed at the Austin abbatoir. The rod form predominated in most of these animals, although the coccus was also present in large numbers.

Four different experiments have been performed to estimate the numbers of cellulose bacteria in one of the experimental animals with a rumen fistula. In the first, there were found 40 million cellulose bacteria per ml. The coccus was the predominating form as determined by the type of colony. The rod was present in the tubes of lower dilution.

It seemed possible that, if the rod required close contact with the cellulose, there could conceivably be present in the rumen similar bacteria which were prevented from growing in agar because of inability to migrate through the gel. Accordingly, a liquid series was tested in parallel with the agar. Serial dilutions were made in the liquid tubes, and from each an inoculation was made also into agar. No significant differences in numbers were given by the liquid and agar series, about 40 million per ml being indicated by each. In this series, also, the coccus was more numerous than the rod.

In a second test the liquid media gave slightly greater numbers than the solid. At least 60 million bacteria per ml were indicated by the liquid series, whereas the agar tubes showed about 10 million.

In the last experiment with the fistula animal, two liquid series were compared, one with sodium thioglycolate and one without. The dilutions were made in the series without the thioglycolate. There were no differences in the number of cellulose bacteria demonstrated by these two series, 100 million per ml being found. This same number was also obtained from a parallel agar series without thioglycolate.

Cellulose was decomposed with great rapidity in liquid tubes inoculated with rumen contents. Tubes inoculated with 1/60,000,000 ml of rumen liquid showed cellulose digestion within 3 days, and within 4 days it had all (10 mg) been decomposed. With 0.001 ml of inoculum, the cellulose was all digested within 24 hours.

During the experiments on culture and isolation, no attention was paid to the problem of obtaining a sample representative of the entire rumen. Much of the rumen contents consists of matted plant material with a high content of solids. It seemed probable that the number of bacteria in this material might be different from the number in the fluid. In order to gain information on this point, a fluid sample was withdrawn from a fistula animal and at the same time some of the matted solid contents were removed. The latter were mixed in a Waring "blendor," and samples were withdrawn after intervals of 1 minute, 6 minutes, and 11 minutes.

The numbers of cellulose bacteria in these samples, and also in the unmixed solid and liquid, were estimated by inoculation into solid and liquid dilution series. There were no significant differences between the agar series inoculated with the liquid rumen contents, with solid material, and with solid material mixed for 1 minute. All showed about 50 million colonies per ml inoculum. No cellulose-digesting colonies appeared in any tube of the agar series inoculated with the samples mixed for 6 and 11 minutes, respectively.

The results in the liquid series were essentially similar. No cellulose digestion occurred in any of the tubes inoculated with the material mixed for 6 or 11 minutes. The liquid and unmixed solid inocula showed growth in the fourth dilution tube. The 1-minute mixed material showed growth in the fifth dilution tube. This indicated 1 billion bacteria per ml. However, too much significance cannot be attached to this greater number, since the three agar series showed little difference and a single organism in the liquid culture would by chance occasionally be carried over.

The failure to obtain any growth at all with the 6- and 11-minute inocula was unexpected. It was improbable that the violent agitation had killed all of the bacteria, since most of the particles of plant material were not broken up to a very great extent. There was no attempt to exclude air during mixing in the Waring "blendor" and a great deal of it was whipped into the material. In view of the great susceptibility of the rumen protozoa to oxygen, it seemed possible that the bacteria were similarly affected.

In the next experiment carbon dioxide was passed into the Waring "blendor" during the mixing. Samples were withdrawn after one-half minute of mixing and after 2 minutes. These were inoculated into both liquid and agar series, as was also the unmixed material. All showed about the same number of cellulose bacteria. The dilutions in these experiments with different inocula were not sufficiently graduated to disclose minor differences in the numbers, but they demonstrated that samples of the rumen liquid give approximately the same number of cellulose bacteria as samples of the solid material.

As a summary of these studies on the numbers of cellulose-digesting bacteria in the rumen demonstrated by cultural methods, it may be stated that a count of approximately 50 million seems to represent the usual number present. In the early studies in which the count was much lower, the technique had not been well worked out. The case in which high counts of over a billion were found are exceptional. Similar high counts have not been found in most of the experiments.

THE SIGNIFICANCE OF THE ISOLATED BACTERIA IN THE RUMEN

The demonstration of anaerobic cellulose digestion by the cocci is in agreement with the conclusions of other investigators on the nature of the cellulose decomposers. Henneberg (1922) correlated the formation of "*Frass-betten*" in cell walls with glycogen formation (iodophily) by the rumen bacteria and concluded, "*Die jodophilen Streptokokken gehören im Wiederkauer zu den wichtigsten Zelluloseverzehrern.*" The cellulose-decomposing cocci which have been isolated apparently correspond to the streptococci which were seen by Henneberg. Baker (1942) has also reported the importance of iodophilic streptococci in the rumen, and it must be concluded that the cultural experiments fully substantiate the findings of these investigators insofar as the cocci are concerned.

Although the isolated cocci have a morphology similar to that of the cellulose decomposers which have been demonstrated by microscopic methods, it cannot be concluded from this evidence alone that the most important organisms have been isolated. Too many bacteria, differing in their cultural requirements, may be gram-negative, iodophilic, cellulose-decomposing cocci, and some of these may not grow under the cultural conditions that have been thus far provided. Additional evidence on the importance of the isolated bacteria can be obtained if the number in which they can be demonstrated culturally is compared to the number demonstrable by microscopic methods.

Rumen contents were diluted 100 times and films were prepared as for the direct microscopic method for counting bacteria in milk. Iodine was first used to stain them, but very few of the bacteria stained intensely. A gram stain was tried but the affinity of the negative cells for the counterstain was so slight that enumeration could not be made. Minute droplets of dye left on the slide after use of Ziehl-Nielsen's carbol fuchsin prevented direct use of this stain. However, it was found that by diluting the carbol fuchsin 50 times the cells were visibly stained, with no deposition of dye except on the bacteria.

One sample of rumen contents from a fistula animal gave a clump count of 8 billion per ml rumen contents. Most of the bacteria were cocci, often occurring in pairs and short chains. Many were slightly elongated and might be classed as short rods. Some minute slender rods were seen and a few large ones. The spiral form was present to the extent of about 300,000 per ml. A few sarcinae were present. Another sample from the same animal at a different time gave 2 billion as the clump count, but the clumps were larger and included more bacteria than the first sample. Otherwise the microscopic appearance was quite similar.

These direct count figures show that the figure of 50 million derived from the culture experiments represents less than 1 per cent of the total bacteria (clumps) present. Under these circumstances, can it be concluded that the isolated cellulose bacteria are representative of the ones active in the rumen? Although the evidence on this point is by no means conclusive, several considerations indicate that the isolated bacteria are actually the ones chiefly responsible for the digestion of the cellulose in the rumen.

(1) The numbers in which they have been demonstrated culturally, while small in comparison with the total, are nevertheless of significant magnitude.

(2) The same kinds of cellulose bacteria, and only those kinds, have been encountered in every dilution series inoculated from rumen contents. If cellulose bacteria with other characteristics were important in the rumen, it seems remarkable that they would not have been at least occasionally encountered.

(3) The cellulose bacteria which have been isolated are extremely active in cellulose decomposition under conditions closely similar to those present in the rumen.

(4) In the thin cellulose agar in the upper part of cellulose dilution tubes inoculated with rumen contents, it is possible to see not only cellulose-digesting colonies, but also noncellulose colonies which develop. By adding water to the tube and loosening the thin agar, it can be dumped out into a petri dish without being torn or mashed. A measured area of uniform thickness can then be cut out, surplus water dried off, and the agar weighed. It is then possible by microscopic examination to obtain not only an idea of the relative number of cellulose and noncellulose colonies, but also of the total number of colonies developing per ml of inoculum.

This was done with a cellulose series inoculated from a fistula animal. Microscopic examination showed that the non-cellulose-digesting colonies greatly outnumbered the cellulose digesters. The proportion was about 1 to 1,000. The total number of colonies was approximately 300 million per ml of rumen contents. This shows that in addition to the cellulose bacteria in the rumen a great many other viable bacteria are present, but not digesting cellulose under the conditions of the experiment. It is probable that the noncellulose bacteria are concerned with the breakdown of hemicelluloses, starches, and soluble materials, with the formation of methane, and with other activities supported by the diverse chemical substances present in the food and derived from the food by the activities of other microorganisms. It is possible that the use of a more fully hydrated and less resistant cellulose than that employed in the present experiments would yield additional numbers of cellulose digesters. However, there is a more or less continuous gradation in the degree of digestibility of various cellulose preparations, and an arbitrary line must be drawn at some point.

The rumen fluid used in the previous experiment had been filtered twice through a Seitz filter. This was necessary in order to remove bacteria and particles which might be mistaken for colonies. Apparently as a result of the filtration, the medium did not support so good growth of cellulose bacteria as in experiments using fresh unfiltered rumen contents, and the number of cellulose bacteria was relatively low. In dilution tubes containing the usual medium an accurate count of the noncellulose colonies could not be obtained, but observations indicated that they greatly outnumbered the cellulose digesters.

Culture series using noncellulose substrates have yielded values of 700 million per ml.

If most of the bacteria seen microscopically are not cellulose decomposers, the numbers in which the cellulose organisms have been demonstrated culturally become more significant and suggest that they are the principal ones concerned.

The significance of the cellulose-decomposing rod in the rumen has not been generally realized. The rod mentioned by Dougherty (1941) is possibly the same. The great activity of the rod in digesting cellulose and its occurrence in significant numbers favor the interpretation that it also plays an important role in the rumen fermentation. The fact the iodophilic reaction is lacking in the rods shows that this feature alone is not an adequate index of cellulose-digesting ability. This might be expected. Materials other than carbohydrates can serve as the substrates from which glycogen is synthesized. The formation of this reserve material depends on the dissimilatory and assimilatory capacities of the organism as well as on the nature of the substrate.

The rumen is an exceedingly complex (and interesting) microcosm and the cellulose bacteria are only one of many groups performing significant functions. However, they are an important group because they participate in the initial attack on an important substrate and provide products which must profoundly influence the course of metabolism of the associated organisms. Knowledge of their activities should aid in understanding the functions of the other organisms.

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SPORE WALL DEMONSTRATED WITH THE ELECTRON MICROSCOPE

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The existence of a resistant wall surrounding the endospores of bacteria is generally accepted. Electronmicrographs of this structure have not been published, to our knowledge.[2]

For this study, a culture of *Bacillus subtilis* was selected from the departmental museum. Its morphological and cultural characteristics agreed with those given for this species in Bergey and in the recent bulletin by Smith, Gordon, and Clark (1946). Transfers were made on ordinary nutrient agar, and cells from cultures of various ages were studied with an RCA electron microscope.

The cell wall was readily demonstrated in apparently healthy cells (figure 1) and in degenerating cells (figure 2). Spores were readily demonstrated inside cell membranes which appeared intact but folded owing to partial collapse around the spore (figure 3). In figure 4 the excess cell wall is seen largely at one end of the spore. Figure 4a is made from the same negative as figure 4 by intensifying the exposure to light in the printing process. A dense wall is seen at either end of the spore. This we interpret as the spore wall. Figures 5 and 5a were similarly made from one negative. With the lesser amount of exposure to light (figure 5) the cell wall is shown; greater exposure to light brings out the spore wall but fails to show the cell wall.

It appears that the main mass of protoplasm within the spore is highly resistant to the penetration of electrons. The less opaque end zones, between the spore wall and this mass, make possible visualization of the spore wall. In the great majority of pictures of spores that were taken, the less opaque zone was not present and the spore wall could not be seen.

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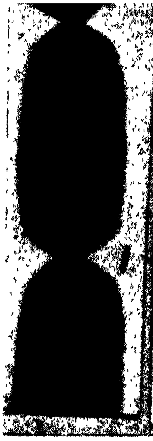
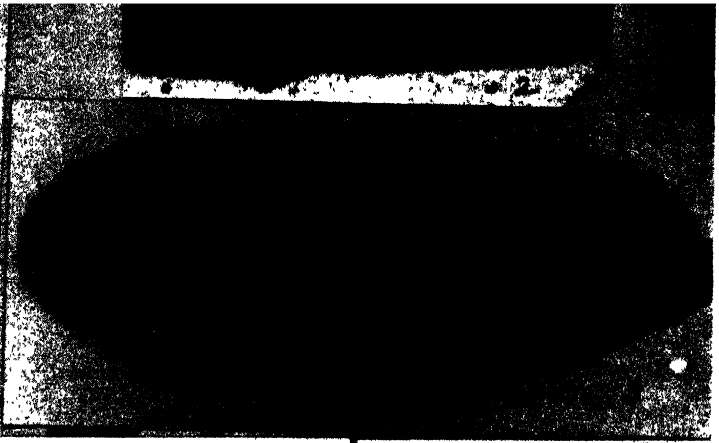
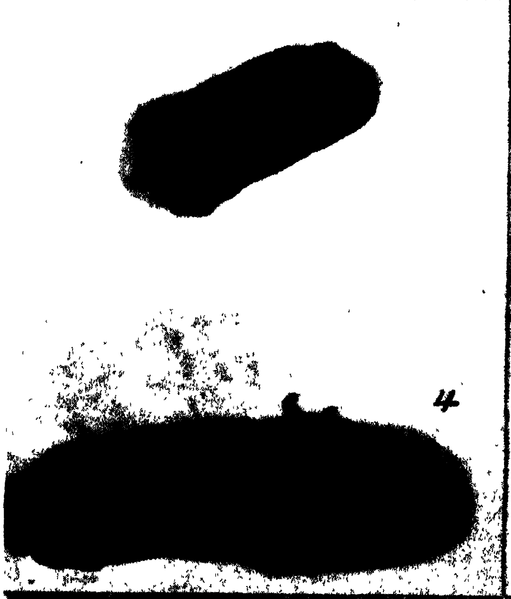
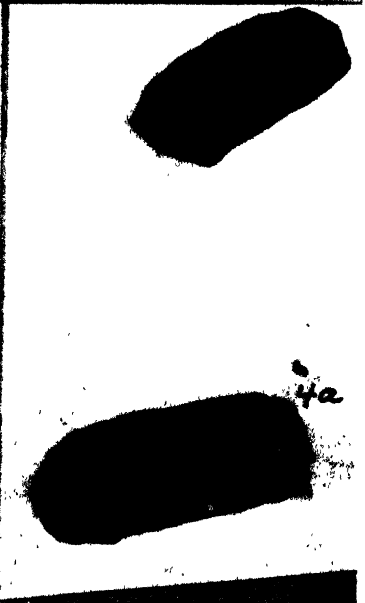
[2] Dr. Georges Knaysi kindly examined these pictures and agreed that the structure shown is the spore wall. He stated that similar pictures of spores have recently been obtained by him and his associates and were to be reported at the meeting of the Electron Microscope Society at Pittsburgh, December, 1946.

[3] (See next page.) It is believed that the preliminary treatment of cells with distilled water, boric acid, formalin, or ethyl alcohol had no significant effect in clarifying or obscuring the structures shown in these pictures.









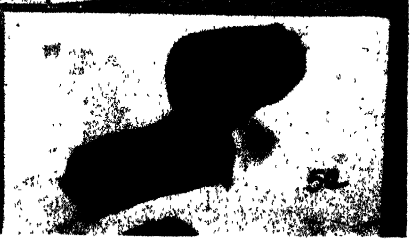


FIG. 1. Twenty-four-hour culture; cells treated with 0.5 per cent boric acid[3] 1 hour. Cell wall is clearly shown surrounding what appears to be healthy protoplasm. × 10,000.

FIG. 2. One-hundred-twenty-hour culture; cells treated with 2.5 per cent formalin[3] briefly. Cell walls are intact. Internal protoplasm has undergone degeneration. Granular precipitate is an artifact. × 10,000.

FIG. 3. One-hundred-twenty-hour culture; cells treated with distilled water[3] 1 hour. Spore is seen inside cell wall (sporangium) that is intact but somewhat folded. × 24,000.

FIG. 4. One-hundred-twenty-hour culture; cells suspended in distilled water[3] and transferred directly to membrane for study. Two spores with cell walls (sporangia) extending beyond one end of cells. × 18,500.

FIG. 4a. Print from same negative as figure 4, but made with greater exposure to light. Spore wall is shown; sporangia are not seen.

FIG. 5. One-hundred-twenty-hour culture; cells treated with 70 per cent ethyl alcohol[3] 1 hour. Two spores are shown inside folded sporangia. × 10,000.

FIG. 5a. Print from same negative as figure 5 made with greater exposure to light. Spore wall is seen at one end of one spore. Sporangia are not seen.

THE ISOLATION AND DISTRIBUTION IN FLORIDA OF AN ANAEROGENIC PARACOLON, TYPE 29911

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The term paracolon has been recommended by Stuart, Mickle, and Borman (1940) to apply to aberrant coliform organisms isolated from man, especially from persons affected with gastroenteritis. In recent years there have been numerous reports regarding the probable pathogenicity of various types of this group of organisms. Edwards (1945) described a paracolonlike bacillus, isolated from colitis in an infant, that possessed antigens typical of the *Salmonella arizona* group. Epidemiologic and bacteriologic data collected by Stuart and Rustigian (1943) indicates that paracolon "bio-type 32011" was the etiologic agent in epidemics of gastroenteritis.

Type 29911 was first described by Stuart *et al.* (1943) in a study of the biochemical and serological relationships of paracolon organisms. Further investigation (Stuart *et al.*, 1946) revealed that the Wakefield type dysentery organisms described by Berger (1945) are identical with this paracolon.

Early in 1945 we isolated a "dysenterylike" organism from a severe case of diarrhea. After unsuccessful attempts to obtain a positive agglutination reaction with available *Shigella* antiserum, the culture was forwarded to Dr. C. A. Stuart, who identified it as paracolon type 29911. In September, 1945, Dr. Stuart requested that we forward to him all cultures, isolated in our laboratory, biochemically resembling type 29911. During the next 3½ months we sent 80 cultures, 63 of which were confirmed as 29911. To date an additional 95 cultures have been isolated in Florida. One of these cultures was obtained from a mild case of diarrhea of 2 weeks' duration in a 2-year-old child.[4]

In view of the evidence found by the authors and by other workers regarding the association of this organism with the diarrheal diseases, the isolation of an unusually large number of cultures in Florida seems to warrant a description of the organisms on the media commonly used in enteric bacteriology, and a note on their distribution in the state.

ISOLATION

The routine procedure for enteric cultures in our laboratories has been described previously (Galton and Quan, 1944); however, a brief review will be given. Fecal specimens received in a glycerol-saline preservative are streaked directly onto SS agar (Difco) and bismuth sulfite (WB) agar (Difco). The amount of fecal material that will adhere to a cotton swab is placed in a tube of Kauffmann's tetrathionate enrichment broth. After 24 hours' incubation the enrichment broth is streaked to an SS agar plate and a brilliant green agar (BG) plate (Kristensen's).

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[3] The authors are indebted to Dr. C. A. Stuart, whose work incited this report, for helpful suggestions and critically reviewing the manuscript. Grateful acknowledgment is also made to Dr. A. V. Hardy and Dr. R. B. Mitchell for advice and criticism.

[4] We wish to thank Dr. H. A. Carithers, Jacksonville, Florida, for information concerning this case.

Of the 158 type 29911 cultures obtained, 63 were isolated from directly streaked SS plates alone; 47 from WB alone; 18 from SS plates from enrichment (SST) alone; 5 from BG alone; 14 from both SS and SST; 9 from both SS and WB; and 2 from SS and BG.

APPEARANCE OF COLONIES

The colonies of 29911 cultures on SS agar appear translucent, smooth, and slightly grayish. Frequently a darker gray ring may be seen about halfway between the center of a colony and the edge.

On WB agar the colonies are not unlike those of *Pseudomonas aeruginosa*—i.e., a greenish brown—differing in that they are usually flat and in well-isolated areas darker brown concentric rings appear.

Although the highest percentage of our cultures were isolated from SS agar, it was found that 29911 strains grow equally well on bismuth sulfite agar, but owing to the similarity of the colonial forms to those of strains of *Pseudomonas* on this medium, which was not known during most of the 3½-month period, they were not picked. After this observation was made, 43 of the 47 cultures isolated on WB alone were obtained. Only 5 cultures were isolated on brilliant green agar, 3 of which were mixed, and pure cultures plated on this medium grew very poorly or failed to grow at all.

DISTRIBUTION IN FLORIDA

These 158 type 29911 cultures were isolated from routine stool specimens received from 31 different cities located from northwest Florida down both the east and west coasts to Key West. From one city health department in the west-central section we found 27 cultures. The remainder were distributed rather evenly throughout the other localities. It is believed that the large number of isolations from this particular town may be attributed, in part, to the greater volume of specimens received from this health unit in comparison to those received from others.

CONCLUSION

Since case histories on our type 29911 cultures were not available, with the exception of 2 isolations, it is impossible to come to any definite conclusion regarding the possible public health significance of these organisms from this group of isolations. No doubt a large percentage were obtained from food handlers, though it is not known whether these persons were free of any enteric complications.

The paracolons may be considered in much the same category as certain members of the *Pseudomonas* and *Proteus* groups and *Shigella alkalescens* regarding their pathogenic status. There is increasing evidence to indicate the implication of these organisms in cases of gastroenteritis and diarrhea. Such paracolon types may eventually be considered as comparable to the *Salmonella* and *Shigella* groups.

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THE ISOLATION OF A STRAIN OF ESCHERICHIA COLI PATHOGENIC FOR THE RABBIT'S EYE FROM A PATIENT WITH DIARRHEA

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In the course of an investigation during which fecal material from patients with diarrhea was inoculated onto the scarified cornea of rabbits, a strain of *Escherichia coli* was recovered. This organism was characterized by its ability to invade the scarified cornea of the rabbit and it appeared to be implicated in the disease of the patient.

Case history. H. R., a 7½-month-old white boy, was admitted to the Cincinnati Children's Hospital November 13, 1944, with a history of cough, respiratory difficulty, and fever of one week's duration. Eight days after admission diarrhea and vomiting began. The stools were numerous, liquid, and contained large amounts of mucus but no blood. Respiratory difficulty, high fever, and diarrhea continued, and for 3 weeks the child had to be fed almost exclusively by intravenous infusion. No specific pathogenic organisms could be cultured from the nasopharynx. The white blood count varied between 9,000 and 10,000 most of the time. The urine was normal except for a moderate albuminuria. The tuberculin test was negative. Cultures of the stools revealed the presence of none of the usual pathogenic bacteria but consistently yielded a colon bacillus, which produced a characteristic lesion when inoculated on a rabbit's cornea. Agglutinins against this organism rose to a high titer in the patient's serum during the course of the disease. Gradual improvement of all symptoms began after the fourth week. The infant was discharged in good condition 2½ months after the onset of his illness.

Reaction of the rabbit's eye. Five stool specimens from the patient, when inoculated onto the rabbit's cornea, produced keratoconjunctivitis (table 1). In each instance a gram-negative bacillus was recovered from the eye in pure culture, and in five tests the pure culture produced panophthalmitis when reinoculated in a second rabbit's cornea. The bacterium was recovered from three of the stool specimens by direct culture on various selective media routinely employed for the isolation of enteric organisms. Inoculation of these pure cultures produced panophthalmitis in rabbits, and the same gram-negative rod was recovered from the eye lesions. Filtrates of the stools produced no corneal lesions.

On about the fourth day after scarification and inoculation an iritis followed by a severe keratoconjunctivitis developed. The reaction remained severe for several days, and then gradually subsided leaving permanent scars on the cornea. Histologic sections of the rabbit's eye revealed a marked cellular infiltration of the cornea, composed mostly of polymorphonuclear leucocytes. The reaction was most intense immediately below the epithelium, particularly in the region of the limbus. The ciliary processes and ciliary muscle were also involved. A cellular reaction, most intense at the base and disappearing toward the margin, occurred in the iris. In the anterior chamber inflammatory cells were also present.

Cultural characteristics of the organism. The organism was a motile, gram-negative rod that produced acid and gas in lactose, glucose, maltose, mannitol, sorbitol, xylose, and rhamnose in 24 hours, and in sucrose, dulcitol, raffinose, and salicin in about 48 hours. Cellobiose, dextrin, and inositol remained unchanged. Milk was rarely coagulated but acid was produced. Gelatin was not liquefied. The organism was methyl-red-positive and Voges-Proskauer-negative. Nitrates were reduced to nitrites, and indole was formed. Small amounts of H_2S were produced in cystine broth. Citrate was not utilized. These reactions are not markedly different from those described for *Escherichia coli* var. *neopolitana* (Bergey, 1939). The culture was tested for agglutination by the various "O" and "H" *Salmonella* typing sera, and it was found to react strongly with the XXXVIII "O" serum but not with other "O" and "H" sera.[1] On the basis of the "IMViC" reactions, the organism is an *Escherichia* belonging to Stuart's group C, type 19 (Griffin and Stuart, 1940).

TABLE 1

Recovery of coliform organism from patient's stool

STOOL NO.	DAY OF DISEASE	STOOL INOCULATED ONTO RABBIT'S EYE			STOOL INOCULATED ONTO MEDIA		
		Reaction in rabbit's eye	Organism recovered from eye	Culture inoculated onto rabbit's eye	Organism recovered	Culture inoculated onto rabbit's eye	Organism recovered from eye
I	13	+	0*				
II	19	+	+	+			
		+	+				
III	24	+	+		+	+	
		+	+	+			
		+	+	+			
IV	33	+	+	+	+		
V	52	+	+	+	+	+	+

* Organism not looked for in this case.

Virulence of the organism. Virulence of the organism was determined by performing intraperitoneal titrations in mice. Tenfold dilutions of an 18-hour culture of the bacteria were prepared in 3 per cent mucin, and 0.5 ml per mouse were injected intraperitoneally into groups of 4 mice each. The LD$_{50}$ titer

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[1] Kindly performed and evaluated by Doctor W. W. Ferguson of the Michigan Department of Health, Lansing, Michigan.

(Reed and Muench, 1938) was $10^{-8.75}$ of a suspension which contained 1,300,000,000 bacteria per ml; hence the probability is that one organism was sufficient to kill a mouse. Without the addition of mucin, only the undiluted culture killed all the mice, and only occasionally did a mouse succumb to a dilution of 1:10. Seitz filtrates of 18-, 48-, and 96-hour broth cultures injected intravenously and intraperitoneally failed to kill mice.

Development of antibodies in patient's serum. Agglutination tests were performed with the bacterium and the patient's serum (table 2). No serum was available during the early phase of the patient's illness. In the serum drawn on the twenty-fifth day, agglutinins were already present in a dilution of 1:640. Their titer rose to 1:1,280 on the forty-second day, and to 1:5,120 on the sixty-seventh day, with partial agglutination at 1:10,240. The patient's serum did not agglutinate known strains of *E. coli* nor a strain of *Aerobacter* which was isolated from his stool. The sera of five other infants with diarrhea failed to agglutinate the coliform organism obtained from the patient. A sixth serum agglutinated the organism in a dilution of 1:40.

TABLE 2

Development of agglutinins in patient's serum

SERUM	DAY OF DISEASE	DILUTION OF SERUM											
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:20,480
I	25	2	4	4	4	4	4	4	1	0	0	0	0
II	42	0	4	4	4	4	4	4	4	2	0	0	0
III	67	0	0	4	4	4	4	4	4	4	3	1	0

0 to 4 indicate degree of agglutination.

DISCUSSION

The organism described appears unusual in its ability to induce a severe corneal reaction. Known laboratory strains of *E. coli* and *Aerobacter aerogenes*, as well as a number of other organisms freshly isolated from diarrheal stools of babies, failed to produce the reaction. Direct inoculation of more than 100 stool specimens of patients with diarrhea likewise failed to produce a corneal reaction, except in the following instances: Twice a lesion of the cornea occurred with the first inoculation, but attempts to isolate an organism or transmit the infection were unsuccessful. Once a strain of *Shigella paradysenteriae* and another time one of *Proteus morgani* were recovered from corneal lesions produced from direct inoculation of stools of infants with diarrhea. In both instances the infection could be transmitted to other rabbits. Several known strains of dysentery bacilli[3] and of *Proteus morgani* failed to produce such a reaction.

Direct evidence is lacking that this strain of *E. coli* was the etiologic agent in the diarrhea of the patient. The organism is a virulent, hemolyzing, and, therefore, presumably necrotizing strain of *E. coli* that has the ability to invade the scarified cornea of the rabbit. The ease with which it was cultured from the patient's stools, the constancy of its occurrence, and the development of a high titer of agglutinins in the patient's serum—all point to multiplication in the intestinal tract and probable invasion of the mucosa.

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[3] Kindly supplied by Doctor Merlin L. Cooper of the Children's Hospital Research Foundation, Cincinnati, Ohio.

SUMMARY

A virulent strain of *Escherichia coli* pathogenic for the rabbit's eye was isolated from the stool of an infant with diarrhea. Presumptive evidence was presented that implicated the organism in the patient's disease process.

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CYTOCHEMICAL MECHANISMS OF PENICILLIN ACTION

I. OXIDATION-REDUCTION LEVELS[1]

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The present investigation was undertaken because it was thought that the results of the experiments might help to elucidate the mechanisms of action of penicillin on susceptible organisms. Although development of new assay methods was not the first objective of the experiments, several of the techniques described below were found to afford reliable and rapid methods of assay that retain the advantages of the cylinder plate technique but eliminate its relatively long incubation period. One of these has already been described (Goyan, Dufrenoy, Strait, and Pratt, 1947). A report of others is in preparation.

Assays of antibiotic agents by means of cylinder plate or cup plate techniques, or their modifications, depend upon a correlation between the concentration of antibiotic in the preparation under test and the diameter of the zone of inhibition that is produced. The zones of inhibition represent areas in which a "static" or "cidal" concentration of the antibiotic exists when the test organisms are in a susceptible stage of growth.

Since there must be a diffusion gradient, outward from the central cylinder or other reservoir of antibiotic, it is reasonable to expect subbacteriostatic concentrations to exist beyond the zones of inhibition. Two observations are of interest in this connection: first, subbacteriostatic concentrations of penicillin have been shown to enhance metabolic activity and growth of *Staphylococcus aureus* (Miller, Green, and Kitchen, 1945; Eriksen, 1946*b*); second, a narrow band of enhanced growth, immediately surrounding the zones of inhibition, is generally evident on test plates and can be seen on published photographs (Eriksen, 1946*b*). A similar zone of markedly enhanced growth immediately surrounding an area of inhibited growth, effected through use of another antibiotic, has been clearly pictured by Burkholder (1944, figure F). In the experiments described below it has been possible to demonstrate by appropriate techniques that these bands of increased growth on penicillin test plates coincide with an area of relatively intense reducing power. It may be suggested that these areas represent regions in which the test organisms have been subjected to subbacteriostatic concentrations of penicillin without subsequent exposure to bacteriostatic, bacteriocidal, or bacteriolytic concentrations, and that, in these areas, the organisms have been stimulated to a state of intense metabolism and growth, perhaps characterized by an abnormally high rate of respiration,

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[1] The execution of the work reported in this paper was made possible by a generous grant from the Cutter Laboratories, Berkeley, California.

[2] With the laboratory assistance of Toinie Juntunen.

such as has been ascribed to the "climacteric stage" induced in cells of various tissues by traces of different chemicals (Hansen, 1946). Of course, these bands of increased growth become much more evident and more easily demonstrable with increased time of incubation. This is probably due to the longer time of exposure to subbacteriostatic concentrations of penicillin. The fact that sharp boundaries can be demonstrated between areas of inhibition and noninhibition suggests that a threshold effect operates in these regions of the plates. We shall present convergent lines of evidence from staining experiments that indicate that such thresholds for a number of chemical groupings can be demonstrated on penicillin assay plates. The experiments reported in the present paper are concerned with tests for oxidizing and reducing substances and systems. Other papers describe the results obtained in tests for different constituents that might be expected to be liberated by the dissociation of cellular complexes and the role of detergents in the mechanism of action of penicillin on susceptible organisms.

The use of the techniques employed in the present work was based on the observation that in normal aerobic respiration of the test organism, as of other organisms, a balance exists between the rate of dehydrogenation of —SH groups effective in hydrogen transfer and the rate of restitution of such groups (Genevois and Cayrol, 1939). Therefore, a deficit of hydrogen donors or of oxygen acceptors eventually develops, and dienol groups become irreversibly dehydrogenated into diketones; concomitantly, the removal of hydrogen provides an opportunity for the development of carbon to carbon linkages, so that dehydrogenated phenolic compounds are likely to polymerize to gummy, quinoid derivatives. Evidence for the presence of quinoid groups will be presented in connection with the indophenol blue reagent.

It is noteworthy also that Wood and Cruikshank (1944) showed the climacteric increase in respiration in plant tissues to be followed by a "decrease in organization resistance," making cell proteins more readily subject to hydrolysis. Thus, at least a partial explanation is suggested for the observation (Gardner, 1940; Fisher, 1946; Eriksen, 1946*a*) that an early evidence of the action of penicillin on susceptible organisms is marked swelling of the cells. The breaking down of cell organization might be expected to expose —SH groups which are normally protected within protein molecules, such as glutathione or the nucleoproteins that play a role in dehydrogenase systems. Such exposed —SH groups should be expected to become more readily accessible to dehydrogenation or to blocking agents. Thus we may have a partial explanation for the fact that, in the presence of detergents, which help unfold protein molecules, and in the presence of an —SH-binding substance such as bismuth, penicillin becomes bacteriostatic at a lower concentration than in their absence (Treffers, 1946; Levaditi and Vaisman, 1946).

MATERIALS AND METHODS

The penicillin used in most of the present work was a crystalline preparation of sodium penicillin G that assayed 1,560 Oxford units per mg. Similar results were obtained with commercial sodium and calcium penicillins assaying about

1,100 to 1,200 units per mg and with an impure preparation of calcium penicillin assaying about 300 units per mg.

The test organisms that were used were *Staphylococcus aureus* NRRL strain no. 313 (the same as FDA strain no. 209P) and the rough form of *Bacillus subtilis* NRRL strain no. B-558. Seeded plates were prepared as prescribed in the specifications of the Food and Drug Administration, which are similar to the recommendations of Schmidt and Moyer (1944). Then they were treated as described below under the individual experiments. In general, after the plates were seeded, one of two procedures was followed before addition of the several stains and reagents: either plates were treated as in the standard FDA assay or they were incubated as for the 3-hour cylinder plate assay described by Goyan, Dufrenoy, Strait, and Pratt (1947). Except as noted below, the procedure used before addition of the dyes or reagents made no qualitative difference in the reactions that were obtained with a given stain or reagent and a given test organism.

EXPERIMENTS AND RESULTS

As long as sources of —SH groups remain in the reduced state, or as long as —SH groups are rehydrogenated as rapidly as they are dehydrogenated there remains in the aerobic cell a reservoir of hydrogen available to rehydrogenate diketones to dienols. Glutathione may be considered an ultimate source of —SH groups. Once all the available —SH groups have been converted to S—S, dienols are no longer protected from irreversible dehydrogenation; on the contrary, they tend to polymerize to more and more irreversibly oxidized quinoids that may tend to catalyze further dehydrogenations. Such a picture of "decompensated aerobic respiration" as might be expected to be brought about by the shift of glutathione, or other sources of sulfhydryl groups, from the reduced to the oxidized condition can be revealed experimentally by evidence obtained from three different but convergent lines of attack that are based on the use of techniques for the detection of reduced —SH or of dienol groups, for the estimation of dehydrogenase activity, and for the estimation of indophenoloxidase activity.

For convenience, the several reagents that were used, the presumed active groups reacting with them, and their effects on different parts of penicillin assay plates are presented in table 1.

Development with Reagents for Reduced Substances

Ferricyanide reagent. Mason (1930) recommended this reagent for the "determination of reduced glutathione in tissues." In this test the reduction of ferricyanide to ferrocyanide is considered to be brought about by the —SH groups, which are thereby dehydrogenated to S—S. Certain other reducing agents may accomplish the same result, but they would not be expected to be encountered in living tissues. Therefore, he suggested that the test makes it possible to locate free sulfhydryl groups as contrasted with disulfide groups and, more specifically, reduced glutathione as contrasted to oxidized glutathione.

When standard assay plates (incubated 16 hours according to FDA specifications) are flooded for 1 minute with a 2 per cent aqueous solution of potassium ferricyanide, drained, and then reflooded for 1 minute with a saturated aqueous solution of ferric sulfate, the uncolored inhibition zones are seen to be bounded by a well-defined deep blue ring which stands out clearly on the less intensely colored general background. The blue color is taken to represent a deposit of Prussian blue where ferri- has been reduced to ferrocyanide. The intensity

TABLE 1

Reagents used in experiments

REAGENT	GROUP ASSUMED TO BE ACTIVE	AUTHORITY*	REACTION ON ASSAY PLATES			
			Color			Definition of boundary†
			Inside of zone	Boundary	Outside of zone	
K-ferricyanide Ferric sulfate	—SH	Mason, H. L. 1930	Faintly bluish	Deep blue	Blue	Very sharp
Cd-acetate	—SH	Binet, L., and Weller, G. 1934; Joyet-Lavergne, Ph. 1938	Clear	Opaque	Cloudy	Not practical
Cobalt nitrate	—SH		Clear	Opaque	Cloudy	Not practical
Schiff's	Aldehyde	Oster, K. A. 1946	Clear	Red	Red	Very sharp
Osmic acid	Dienol (*o*-polyphenols)	Dufrenoy, J. 1945	Clear	Black	Smoke, gradually darkening to black	Very sharp
Chromaffin 1 {K-bichromate Hg-bichloride}	Dienol (*o*-polyphenols	Lison, L. 1936	Clear	Orange	Brown	Sharp
2 {NH_4-molybdate in acetic acid}	Dienol (*o*-diphenols)	Lison, L. 1936	Clear	Dark brown	Brown	Very sharp
$FeCl_3$	Dienol (*o*-diphenols)	Lemoigne, M. 1928	Clear	Green	Green	Not practical
Azo reaction (in alkaline solution)	Dienol (*o*-polyphenols)	Lison, L. 1936	Faintly pink	Vivid red	Red	Sharp
Methyl green Malachite green Janus green	Dehydrogenase systems	Prevot, A. R. and Ferly, A. 1946	Green		Faintly pink	Moderately sharp
"Thydi" (*p*-phenylenediamino chlorhydrate and thymol, buffered at pH 9)	Indophenol oxidase	Lison, L. 1936	Blue		Clear	Not practical

* For complete citation see list of references at the end of the paper.

† Reagents that give boundaries designated as "sharp" or "very sharp" are considered of potential value in the development of rapid cylinder plate methods of assay. Those designated "not practical" are useful in experimentation but are considered unsuitable for routine work.

of color indicates the relative abundance of reducing substances. *S. aureus* plates after 3 hours of secondary incubation, as described in a previous paper (Goyan *et al.*, 1947), and *B. subtilis* plates after 2 hours of secondary incubation can be developed promptly by similar treatment. This technique gives a very sharp definition of the inhibition zones.

Cadmium acetate reagent. Standard assay plates treated with a solution of 1 per cent cadmium acetate rapidly develop a densely opaque ring, such as might be expected to evidence the formation of cadmium glutathionate immediately around the clear zone of inhibition. This ring is coincident with the ring of enhanced growth surrounding the zone of inhibition and suggests that in this region glutathione is present in the reduced condition. Glutathione can also be detected through the formation of complexes with salts of other metals, such as cobalt nitrate.

Schiff's reagent. This reagent, prepared by decolorizing a hydrochloric solution of fuchsin with sodium sulfite, has been used to detect the presence of aldehydic groups. When plates seeded with *B. subtilis* and incubated 3 hours or more with penicillin are flooded with the sulfite-fuchsin mixture, a deep red color develops immediately in the background and clearly outlines the unstained inhibition zones. A sharp delineation of the zones is produced on plates seeded with *S. aureus* and flooded with this reagent, however, only when the plates are first pretreated with a 1 per cent aqueous solution of $HgCl_2$, according to the procedure recommended by Oster (1946), to liberate bound aldehydes. We believe the color developed on the penicillin test plates is to be ascribed to the restoration of basic fuchsin where aldehyde groups exist freely (as on plates seeded with *B. subtilis*) or are liberated by $HgCl_2$ (as on plates seeded with *S. aureus*). It should be pointed out, however, that the development of the red color outside the zones of inhibition might also be ascribed to adsorption phenomena, which will be discussed in another paper. Colonies can be stained red by the sulfite-fuchsin mixture, and also on plates which have been submitted to physical development according to the procedure described previously (Goyan *et al.*, 1947).

The techniques which are considered in this paper differ from that of physical development, reported earlier, in that physical development involves reactions in both the agar medium and the bacterial colonies, whereas in the present techniques the bacterial colonies are stained without any staining action on the agar. Thus the present techniques may be truly considered cytochemical, since they involve only the cells themselves.

Development with Reagents for Phenolic Compounds

The preceding sections show that the areas inside the zones of inhibition differ from the areas outside in failing to give a positive response for —SH groups. Since phenolic compounds within the organisms can be expected to be protected from irreversible dehydrogenation only as long as —SH groups are available, a threshold for diketones vs. dienols should coincide with that for S—S vs. —SH. By diketones vs. dienols we mean the active groups of quinoid derivatives vs.

those of the original *ortho*-diphenols. Evidence for such a threshold can be revealed experimentally by several techniques that have been discussed by Lison (1936) as to their specificity in revealing prescribed active groups. These techniques may be classified as involving (a) argentaphil (or "argyrophil"), osmiophil, and chromaffin reactions, and (b) azo reactions. The argentaphil, osmiophil, and chromaffin reactions have been used for many years for histochemical studies.

Argentaphil. A method of silver impregnation (essentially an argentaphil reaction) has been described previously (Goyan *et al.*, 1947) and will not be reviewed here.

Osmiophil. It should be recalled that the osmiophilic reaction, often incorrectly ascribed to fats, indicates the reduction of osmic acid through the mediation of reducing agents. (In living cells these are likely to be phenolic compounds.) The development of plates by exposing them to the fumes of osmic acid, therefore, may depend fundamentally on the same mechanisms, i.e., processes of reduction that are brought into operation in the method of physical development previously reported, and which account for the precipitation of metallic copper outside the inhibition zones when plates are flooded with Fehling's reagent.

In our experiments, after the cylinders were removed from the plates a few drops of a 2 per cent aqueous solution of osmic acid (stabilized by chromic acid) were placed in the cover of the petri dish, the bottom was replaced, and the dish was kept in an inverted position. When this is done, within a few minutes dark rings outline the outer margins of the clear inhibition zones, thus revealing the zones of enhanced metabolic activity where the test organisms might be expected to be at the climacteric stage. Progressively, the blackening extends to the background, outside the inhibition zones, and, of course, eventually even the inhibition zones themselves darken. As in other methods of development, exposure to the reagent must be timed properly to give the sharpest definition. An advantage of this method for research purposes is that it eliminates the danger of mechanically washing colonies off the test plates.

Chromaffin reaction. Lison (1936) pointed out that the chromaffin reaction, which involves dehydrogenation of phenolic compounds in the tisue itself to colored quinoid derivatives, is the only one which can be considered specific for *ortho*-diphenols in histochemical or cytochemical work. At the boundary of the inhibition zone, a region of enhanced growth, the organisms might be expected to be most active in synthesizing and storing phenolic compounds. These would be likely to yield colored derivatives under the action of mild oxidants, such as are employed in the so-called chromaffin reaction.

A chromaffin reaction is obtained easily by flooding the assay plates with a mixture of a saturated aqueous solution of potassium bichromate and mercury bichloride containing 10 per cent formaldehyde, whereupon a conspicuous orange ring promptly develops around the clear inhibition zones. Gradually the entire background outside the inhibition zones colors while the interiors of the zones remain relatively uncolored.

An even sharper definition of inhibition zones may be observed when plates are treated with a saturated solution of ammonium molybdate in acetic acid. In animal tissues a similar color reaction with this reagent has been ascribed to the formation of a deep brown complex between ammonium molybdate and *ortho*-diphenols. The reaction is not specific, however, since conceivably it might be merely the result of the dehydrogenation of those diphenols to the corresponding brown quinoids.

In 1928 Lemoigne reported that *B. subtilis* produces an *ortho*-phenolic compound that yields a colored derivative with saturated solutions of $FeCl_3$. We have observed that when assay plates seeded with *B. subtilis* are flooded with a saturated solution of $FeCl_3$, green rings that outline the zones of inhibition appear promptly as the general background of the plate outside the inhibition zones darkens. The color develops more vividly and persists longer if a dilute solution (about 1 per cent) is employed. This is especially true for plates seeded with *S. aureus*. It is well known that *ortho*-diphenols, such as catechol, form colored complexes with $FeCl_3$. This reaction, however, is not unequivocal evidence of the presence of such phenolic compounds, since various other molecular structures that might be present in living tissues (such as hydroxamic acids, for example) may also give rise to colored complexes with $FeCl_3$.

Azo reaction. Lison (1936) described a technique which has been considered specific for the determination of intracellular phenolic compounds. The reaction depends upon the formation of an azo dye when phenols react with the reagent, which consists of a freshly prepared, cold, alkaline solution containing an aromatic amine and sodium nitrite. We have used naphthylamine. When assay plates seeded with *S. aureus* are flooded with such a reagent, the general background almost immediately develops a bright red color which sharply outlines the relatively unstained zones of inhibition.

Development with Reagents for Dehydrogenase Activity

Reactions obtained with ferricyanide and with cadmium acetate have been discussed as evidence of threshold effects with respect to —SH groups within and without the zones of inhibition. Reactions obtained with reagents for phenolic compounds have been discussed as evidence of threshold effects with respect to the presence of dienols outside the zones as contrasted to their absence inside the zones. These results can be correlated with evidence for decreased dehydrogenase activity and correspondingly increased oxidase activity within the zones.

When assay plates seeded with *S. aureus* are flooded with a 0.1 per cent aqueous solution of methyl green, the inhibition zones immediately appear as vivid green areas on a faintly pink background. This can be interpreted to mean that the dye fails to be reduced by the organisms under the influence of bacteriostatic concentrations of penicillin, but that it is promptly reduced to the pink or leuco derivative by those cells outside the sphere of inhibiting concentrations. Experiments with methyl green have been cited as an example, but other dyes (malachite green, Janus green, etc.) currently used as rH indicators may be used to detect the upward shift of rH where colonies of *S. aureus* have been exposed to the bacteriostatic action of penicillin. This is in contrast to the preservation of the normal aerobic level of oxidation-reduction potential outside the range of diffusion of bacteriostatic concentrations.

Similarly, results obtained with various pH indicators suggest that the zones of inhibition are sites of a downward shift in pH from approximately 7 to pH 6 or lower. It should be pointed out, however, that such staining reactions or changes of color may also be interpreted as manifestations of a differential adsorption of molecules or of ions in the inhibition zones and in the background. Evidence concerning the interference of surface effects will be presented in another paper, dealing with adsorption phenomena. In the present paper we are confining our attention to converging lines of evidence which seem to suggest a lessening of dehydrogenase activity, and a concomitant increase of oxidase activity inside the inhibition zones.

Although impairment of dehydrogenase activity can be inferred from the results of a diversified set of reactions, the only biochemically reliable test for oxidase appears to depend on the use of freshly prepared "thydi" reagent. When plates are flooded with this reagent, nascent indophenol blue develops more rapidly and more intensely in the larger zones of inhibition, around cups containing higher concentrations of penicillin, and correspondingly more slowly and less intensely in the smaller zones, surrounding cups containing lower concentrations. The formation of indophenol blue may be considered to be catalyzed by quinoid derivatives arising from irreversible oxidation of phenolic compounds by phenoloxidases. Phenoloxidases are known to remain active after cellular organization has been destroyed. Consequently, they might be expected to remain active in regions where dehydrogenases have been dispersed or otherwise inactivated. Dehydrogenases are known to be inhibited by various agents at concentrations far below lethal levels (Bach and Lambert, 1937, 1938).

DISCUSSION AND CONCLUSIONS

All of the techniques reported in this paper intensify the boundaries between areas of inhibition and noninhibition on penicillin assay plates, whether the zones are clearly discernible without further treatment (as in the standard FDA test) or are very faintly visible (as after only 2 or 3 hours of incubation and diffusion of penicillin). The techniques that have been described all agree in locating the margin of the zone of inhibition at the same distance (within the limits of experimental error) from a cylinder from which a given concentration of penicillin has been permitted to diffuse for a given length of time. This is of some practical interest since it opens up several new methods of conducting rapid assays for penicillin without the use of expensive or elaborate equipment.

The theoretical implications of these techniques are of greater interest, however, since they all provide convergent lines of evidence that may be interpreted to mean that a threshold effect obtains on penicillin assay plates and that that effect depends, in part at least, upon an oxidation-reduction threshold. The sharp boundaries at the margins of the inhibition zones may be an expression of the shift of —SH to S—S. Reagents stated to be specific for —SH groups in living cells and tissues failed to reveal such groups in the zones of inhibition but indicated their presence outside the zones. Several reagents for di- and polyphenols indicated their relative abundance in the areas of normal growth and their relative scarcity in the areas of inhibited growth. Other reagents for detecting the activity of dehydrogenase systems indicated that, in comparison with areas of normal growth, the areas of inhibited growth were deficient in dehydrogenase activity. This is pertinent, since it is well known that many dehydrogenase systems depend for their activity in hydrogen transfer on the presence of reduced sulfhydryl groups. Use of the "thydi" reagent indicated a higher oxidase activity in areas of inhibition than in areas of normal growth.

Koschtojanz and Turpajew (1946) stressed the potential importance of sulfhydryl groups in the processes of irreversible denaturation of protein structures under the conditions prevailing in a living cell. In another paper we shall present cytochemical evidence of dissimilation of nucleoprotein complexes in bacterial cells under the influence of penicillin, and we will show how these processes are intimately related to the sulfhydryl-disulfide picture that is indicated by the present work.

SUMMARY

A study of penicillin assay plates has been made by means of techniques intended to delineate the cytochemical picture that develops on such plates when the test organisms are subjected to the action of penicillin.

The present paper is concerned with techniques intended to yield information about oxidation-reduction levels in different parts of the plates.

All of the tests that were used (14) agreed in indicating that the zones of inhibition are characterized by relatively high levels of rH as compared with the areas of noninhibited growth.

Evidence is presented to show that the very sharp demarcation between areas of inhibition and of normal growth is due to the development of a narrow band of increased growth and metabolism immediately outside the zones of inhibition. This band is attributed to the stimulation of cells subjected to appropriate subbacteriostatic concentrations of penicillin without subsequent exposure to concentrations able to induce stasis, death, or lysis.

The sharp separation of the zones from the background on test plates is taken as evidence for a threshold effect.

The patterns developed on the plates subjected to the different stains and reagents are interpreted largely in terms of relative concentrations of —SH and S—S groups.

Several of the techniques employed are adaptable as new procedures for rapidly assaying penicillin by the cylinder plate method.

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NOTES

PARACOLON ORGANISMS IN SPRAY-DRIED WHOLE EGG POWDER

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In the examination of spray-dried whole egg powder (4 to 6 per cent moisture) for *Salmonella*, a number of organisms were isolated whose morphological and biochemical characteristics were similar to those of strains of *Salmonella* except that they fermented lactose slowly. The same preliminary criteria were used for screening out all but the salmonellae. Thus, the organisms selected for further study had the following basic characteristics: lactose +, sucrose −, H_2S +, indole −. Approximately 164 cultures isolated from as many samples of powder fell into this classification. They were further divided into (a) those giving reactions with the 5 basic *Salmonella* "O" group sera (B, C_1, C_2, D, and E), of which there were 47 cultures, and (b) those failing to give reactions with any of the 5 group sera, of which there were 117 cultures. The former group of 47 cultures has been set aside for further study. The latter paracolons were forwarded to P. R. Edwards (Lexington, Kentucky, Salmonella Typing Center), who found them to be antigenically related to the "Arizona" group. Together with organisms isolated from other sources, they were utilized by Dr. Edwards and his associates as a basis for an antigenic classification of the "Arizona" group (in press). This report contains information regarding the paracolon strains within the "Arizona" group which were isolated from spray-dried whole egg powder.

The 117 paracolons referred to represent 14 of Edwards' 19 antigenic groups. Eighty-seven of the strains fell into four groups; i.e., 15 into group 1, 16 into group 2, 19 into group 6, and 35 into group 7. The remaining 30 strains were distributed in groups 3, 5, 8, 9, 10, 11, 13, 15, 18, and 19.

The paracolons were isolated from powder received from 29 dehydration plants located within 16 states. It is evident, therefore, that their distribution was widespread. One plant, however, contributed a total of 31 paracolons, representing 9 of the antigenic groups. This plant also contributed the greatest variety of *Salmonella* types.

There was approximately a 1:10 relationship of paracolons to the salmonellae isolated from egg powder. If one were not limited to a specific biochemical configuration (i.e., lactose +, sucrose −, H_2S +, indole −) for selection of organisms for consideration, there is no doubt that as a whole paracolons would be found in greater numbers in egg powder.

This study has demonstrated that high-moisture (4 to 6 per cent) egg powder manufactured from unpasteurized liquid egg is a good source material for the isolation of paracolons. In view of the increasing evidence of their pathogenicity, it is suggested that further study of the paracolon problem be undertaken.

THE SUSCEPTIBILITY OF CROSSBRED MICE TO POLIOMYELITIS VIRUS

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As a result of an accidental cross between a Swiss white female mouse and a wild gray mouse frequenting our animal room, offspring were produced that proved to be markedly susceptible to the virus of poliomyelitis. The female mouse was nursing a litter of purebred Swiss white mice when she escaped from her cage on several occasions. Approximately 6 weeks after this series of escapades, she gave birth to a litter of 9 gray mice. The offspring were larger than those of another litter of 8 purebreds born on the same day. Also they gained weight more rapidly and otherwise appeared to be more sturdy. When both litters were 5 weeks of age, they were injected intracerebrally with 0.03 ml of a brain-cord suspension of the Lansing virus. Fifty per cent of the crossbred mice were paralyzed or dead of the infection within 4 days, as compared to 7 days for the purebreds. All the crossbreds had succumbed to the infection within a week, whereas 3 of the purebreds failed to show evidences of infection although kept until a month after inoculation.

Although it might be exceedingly difficult, under the usual circumstances of breeding laboratory livestock, to mate Swiss white and wild gray mice, our experience introduces the possibility that crossbreeding may produce a valuable laboratory animal for the cultivation of viruses to which the purebred white mouse is relatively insusceptible.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

WASHINGTON BRANCH

WASHINGTON, D. C., JANUARY 28, 1947

PRESERVATION OF CERTAIN MICROORGANISMS UNDER PARAFFIN OIL. *Ruth E. Gordon*, American Type Culture Collection, Washington, D. C., *and Nathan R. Smith*, Plant Industry Station, Beltsville, Maryland.

The method of preserving cultures of microorganisms under sterile paraffin oil is being tested as an additional means of maintaining our strains. The technique is very simple. Cultures showing a maximum amount of growth on short agar slants or stabs are covered with sterile oil to a depth of at least one-half inch. Viability is determined approximately every 6 months by transferring some of the growth under the oil to a fresh tube of a suitable medium. Incomplete results indicate that the method can be used successfully with cultures of the following genera: *Achromobacter*, *Actinomyces*, *Agrobacterium*, *Bacterium*, *Brucella*, *Caseococcus*, *Cellulomonas*, *Chromobacterium*, *Coccobacillus*, *Corynebacterium*, *Eberthella*, *Erysipelothrix*, *Escherichia*, *Flavobacterium*, *Gaffkya*, *Klebsiella*, *Kurthia*, *Listerella*, *Microbacterium*, *Micrococcus*, *Mycobacterium*, *Mycoplana*, *Neisseria*, *Pasteurella*, *Phytomonas*, *Propionibacterium*, *Proteus*, *Rhizobium*, *Saccharomyces*, *Sarcina*, *Serratia*, *Shigella*, *Spirillum*, *Torula*, and *Xanthomonas*.

The method was unsatisfactory for some cultures of the following genera: *Aerobacter*, *Alcaligenes*, *Cytophaga*, *Erwinia*, *Leuconostoc*, *Pseudomonas*, *Staphylococcus*, and *Vibrio*.

UNIDENTIFIED ESSENTIAL GROWTH FACTORS FOR LACTOBACILLUS LACTIS FOUND IN REFINED LIVER EXTRACTS AND IN CERTAIN NATURAL MATERIALS. *Mary S. Shorb*, Division of Nutrition and Physiology, Bureau of Dairy Industry, Agricultural Research Administration, U. S. Department of Agriculture.

Lactobacillus lactis Dorner (ATCC 8000) failed to grow in an amino acid basal medium containing all of the synthetic B vitamins, or when the medium was supplemented with either clarified tomato juice or certain preparations of liver extracts. Maximum turbidity and acid formation were obtained when the tomato juice and liver preparations were added together. An assay method was developed for each factor. The liver factor (LLD) was found in high concentrations in refined liver extracts and in lower amounts in Wilson liver fraction L, brewers' yeast, liquid skim milk, pepsin, erepsin, unclarified tomato juice, and in bacto yeast extract, autolyzed yeast, trypsin, peptone, tryptone, tryptose, and peptonized milk. These materials had some tomato juice factor (TJ) activity. Crude casein, calcium caseinate, alcohol-extracted casein, acid-hydrolyzed "vitamin-free" casein, tryptic digests of crude or "vitamin-free" casein, zinc insulin crystals, and chymotrypsin were inactive for LLD but possessed TJ activity. Insulin, chymotrypsin, and some tomato juices also showed some inhibitory action. Phosphopeptone, ascorbic acid, yeast nucleic acid, sodium salt of thymonucleic acid, and adenylic acid were inactive for both factors. A third factor is synthesized. LLD does not appear to be identical with any of the unidentified growth factors for bacteria in distribution and properties. The other factor(s) may be related to certain bacterial and animal growth factors.

EASTERN PENNSYLVANIA CHAPTER

ONE HUNDRED AND NINETY-FIRST MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, PHILADELPHIA, PENNSYLVANIA, JANUARY 28, 1947

THE EFFECT OF CARONAMIDE UPON PENICILLIN THERAPY OF EXPERIMENTAL PNEUMOCOCCUS AND TYPHOID INFECTIONS IN MICE. *W. F. Verwey and A. Kathrine Miller*, Department of Bacteriology, Medical Research Division, Sharp and Dohme Laboratories, Glenolden, Pennsylvania.

The oral administration of 4′-carboxyphenylmethanesulfonanilide (caronamide) was found to enhance considerably the therapeutic effectiveness of intramuscularly administered penicillin in mice experimentally infected with either type I pneumococci or *Eberthella typhosa*. The enhancement of penicillin activity was 6- to 16-fold in the pneumococcus experiments and approximately 4-fold in the *E. typhosa* experiments. The effect of caronamide is the production of higher and more prolonged plasma penicillin concentrations in the animal rather than a direct synergistic action between the drug and penicillin. Caronamide is believed to act by inhibiting specifically the enzymatic transport mechanism involved in the tubular excretion of penicillin.

A MECHANISM FOR THE DEVELOPMENT OF RESISTANCE TO STREPTOMYCIN AND PENICILLIN. *Morton Klein*, Department of Bacteriology, University of Pennsylvania, Philadelphia, Pennsylvania.

A study was made for the presence in broth cultures of highly resistant variants to penicillin and streptomycin in order to determine the correlation between the incidence of resistant variants and the rate of development of drug resistance.

From 40 ml of a 24-hour broth culture containing approximately 8 x 10^9 bacteria, a 0.4-ml sample was tested for initial drug susceptibility. The presence of a few bacteria in the total 40 ml of culture having a high degree of drug resistance was determined by seeding the total 40 ml (in ninety-nine 0.4-ml samples) into broth containing drug concentrations 5, 10, and 250 times the concentration inhibiting the growth of the single 0.4-ml sample. Growth in a tube indicated the presence of a variant resistant to the high drug concentration. In seven 100-tube assays, using 6 gram-negative and gram-positive strains, a total of 57 variants were found showing a high degree of streptomycin resistance. In a total of 18 penicillin assays, using 8 strains, no highly resistant penicillin variants were found. The presence of highly resistant streptomycin variants was correlated with the rapid development of streptomycin resistance.

ONE INSTANCE OF THE FAILURE TO REMOVE SENSITIZING MATERIAL FROM A BERKEFELD FILTER IN SPITE OF CAREFUL CLEANSING. *Ida Teller*, Section of Allergy, Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania.

Eleven hundred ml of ragweed pollen extract were filtered through a 5-by-1-inch Berkefeld filter. The filter was then cleaned by boiling in sodium carbonate solution and in tap water three times and by rinsing with tap water before and after each boiling. It was then rinsed with distilled water and autoclaved. Afterward it was used to sterilize some extracting (Coca's) fluid. The first 15 to 20 ml that came through the filter had a yellow color characteristic of fresh ragweed extract. A ragweed-sensitive patient was tested by the intracutaneous method with this material and with a control of Coca's fluid which contained no ragweed. The former material, which we suspected had some ragweed in it, gave a positive reaction with a large area of wheal and erythema, whereas the control Coca's fluid showed only a small wheal and very little erythema. The fluid which came through the filter after cleansing and autoclaving, therefore, contained traces of ragweed.

HISTOPLASMIN SENSITIVITY AND NONTUBERCULOUS PULMONARY CALCIFICATIONS. *Robert H. High*, Department of Pediatrics, School of Medicine, Temple University, Philadelphia, Pennsylvania.

MICHIGAN BRANCH

DETROIT, MICHIGAN, FEBRUARY 6, 1947

THEORY AND PRACTICE OF MICROBIOLOGICAL ASSAYS OF B COMPLEX VITAMINS. *A. P. Bradie*, The Difco Laboratories, Detroit, Michigan.

Microbiological methods of assay of B complex vitamins are based on the fact that these substances are essential nutrilities for many bacteria. Furthermore, within certain limits, the response of the test organism as measured by growth or acid production is directly proportional to the concentration of the vitamin present. The use of dehydrated basal media was discussed.

A TURBIDIMETRIC METHOD FOR THE ASSAY OF ANTIBIOTICS. *Dwight A. Joslyn and Margaret Galbraith*, Research Laboratories of Parke, Davis and Company, Detroit, Michigan.

GENE RECOMBINATION IN THE BACTERIUM ESCHERICHIA COLI

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The study of inheritance in bacteria has, for the most part, been confined to the investigation of mutational changes in the course of clonal reproduction. With the exception of experiments on pneumococcus type transformations there have been few studies on the direct hereditary interaction of one bacterial type with another. The conception that bacteria have no sexual mode of reproduction is widely entertained. This paper will be devoted to the presentation of evidence for the occurrence in a bacterium of a process of gene recombination, from which the existence of a sexual stage may be inferred.

On the basis of mutation studies many investigators have concluded that the hereditary properties of bacteria are based on the existence of genes (Luria and Delbrück, 1943; Roepke *et al.*, 1944; Lwoff, 1941; Demerec and Fano, 1945; Gray and Tatum, 1944), although it is not clear whether these genes should be homologized with the Mendelian factors of higher organisms, or with the extranuclear factors which have been demonstrated in some microorganisms and higher plants (Sonneborn, 1943; Spiegelman *et al.*, 1945; Rhoades, 1943).

The genic basis of microbial inheritance does not depend on the demonstrability of a sexual phase in bacteria. However, more powerful genetic methods paralleling classical Mendelian analysis would be available if it were possible to follow the inheritance of characters in the products of a sexual fusion. The few examples of this approach thus far reported have provided no incontrovertible evidence for sexual reproduction in bacteria.

The phenomenon of paragglutination in the colon-typhoid-dysentery group might be regarded as an instance of bacterial hybridization, and was so interpreted by Almquist (1924). As reported by numerous authors, paragglutination refers to the development of new types which react with antisera for each of two distinct strains when these are grown together in mixed culture (Kuhn and Ebeling, 1916; Salus, 1917; Wollman and Wollman, 1925). The significance of these observations has been attacked by several authors (Breinl, 1921; Arkwright, 1930; Kauffmann, 1941), chiefly on the grounds that the paragglutination represents a nonspecific cross reactivity characteristic of "rougher" phases of these organisms. Hansen (1929) failed to obtain paragglutination in her experiments. In the light of more detailed recent information on the antigenic structure of this group, this problem certainly deserves a critical reinvestigation.

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[1] Fellow of the Jane Coffin Childs Memorial Fund for Medical Research. This work was supported in part by a grant from the Jane Coffin Childs Fund, and will be submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Yale University.

Sherman and Wing (1937) have described experiments designed to detect recombinations of fermentative characters in mixed cultures of various *Escherichia coli* and *Aerobacter aerogenes* strains. Although new combinations of biochemical characters were found, similar types were found to an equal extent in pure cultures, so that these authors could not infer the occurrence of a sexual fusion. Their experiments are of the greatest interest, however, since they represent the first attempt to study this problem in bacteria by genetic methods using clear-cut characters. Gowen and Lincoln (1942) later performed similar experiments with strains of *Phytomonas stewartii*, using cultures differing in morphological and pigment characteristics. As in Sherman and Wing's studies, these authors were unable to differentiate the new types they found in their mixed cultures from types which arose spontaneously in single cultures. For this reason a definite conclusion could not be drawn from their results.

A discussion of hereditary processes in bacteria must take into account the extensive work on transformation of pneumococcal types, first described by Griffith (1928) and culminating in the isolation of the transforming principle in chemically characterizable form by Avery, MacLeod, and McCarty (1944). These studies have revealed that, under special experimental conditions, a product isolated from a serologically specific, smooth, pneumococcus culture will convert cells of a nonspecific rough culture to the smooth type characteristic of the source of the transforming principle. So far as is known, such transformations can be performed in only one direction (rough to smooth) and only under very special conditions. Boivin and Vendrely (1946) have reported a similar transformation involving the capsular polysaccharide of a strain of *E. coli*. There have been reported other instances of varying credibility (Kasarnowsky, 1926; Lommel, 1926; Legroux and Genevray, 1933; Frobisher and Brown, 1927; Burnet, 1925; Holtman, 1939; Cantacuzene and Bonciu, 1926). These studies have a direct bearing on recombination experiments, since transformations of this sort might be responsible for the occurrence in mixed cultures of some new types which are interpretable as recombination types. This will be discussed in more detail below.

Morphologically unusual forms of various bacteria have been described by Mellon (1925) as zygospores, and been taken to imply a sexual fusion. It has been suggested by Dienes and Smith (1944) and by Smith (1944) that the "Large Bodies" which appear under certain conditions in cultures of *Bacteroides* may represent a sexual phase. There is as yet no evidence that the recombination phenomena in *E. coli* which will be discussed in this paper are related to any special structural form, such as those which have been described by these authors.

MATERIAL AND METHODS

Except as otherwise stated, the experiments reported on in this paper have all been performed with mutants of a single strain of *Escherichia coli*, K-12. This is a typical coliform bacterium originally isolated from human feces—a gram-negative rod, motile, lactose-fermenting, producing indole, and susceptible to each of the coli phages in the series T1 to T7 of Demerec and Fano (1945).

It has been used at Stanford University as a student laboratory strain for a number of years.

Mutant strains of *E. coli* characterized by specific growth factor requirements have been obtained after treatment with X-rays, ultraviolet light, and nitrogen-mustard. A single nutritional requirement is established at a single mutational step, and on the basis of studies on *Neurospora* is regarded as based on a change in a single gene. By successive treatments, multiple mutant strains with several genetically and biochemically independent nutritional requirements have been produced. The strains used in these experiments are described in table 1. In general, the nutritional characteristics of a strain are ascertained by inoculating media consisting of the basal medium plus various supplements; lack of visible growth in the absence of a given growth factor and optimal growth in its presence are the criteria for the determination of the nutritional requirements of a strain.

TABLE 1

*Characteristics of Escherichia coli biochemical mutants**

STRAIN	REFERENCE†	REQUIREMENTS	SYMBOL	OBTAINED FROM	TREATMENT	DETECTION TECHNIQUE‡
58	1	Biotin	$B-$	K-12	X-ray	1
679	1	Threonine	$T-$	K-12	X-ray	1
58-161	2	Biotin, methionine	$B-M-$	58	X-ray	1
679-680	2	Threonine, leucine	$T-L-$	679	X-ray	1
Y10	3	Threonine, leucine, ✓ thiamine	$T-L-B_1-$	679-680	X-ray	2
58-278	2	Biotin, phenylalanine	$B-\phi-$	58	X-ray	1
Y24	3	Biotin, phenylalanine, cystine	$B-\phi-C-$	58-278	Ultraviolet	2
Y38	3	Arginine	$A-$	B/r§	Ultraviolet	2
Y39	3	Histidine	$H-$	B/r	Ultraviolet	2
Y44	3	Arginine, methionine	$A-M-$	Y38	Ultraviolet	2
Y45	3	Histidine, *p*-aminobenzoic acid	$H-Pb-$	Y39	Ultraviolet	2
679-183	2	Threonine, proline	$T-P-$	679	X-ray	1

* Mutants for resistance to phage T_1 have been obtained in strains 58-161, 679-183, Y10, and Y24, without detectable variation in nutritional requirements.

† References: (1) Gray and Tatum, 1944; (2) Tatum, 1945; (3) previously unpublished.

‡ Technique 1 is described by Gray and Tatum (1944); 2 by Lederberg and Tatum (1946).

§ This is a radiation-resistant mutant of *E. coli* B, isolated by Witkin (1946).

A mutant strain can be signified by suffixing a "−" sign to the initial of the substance in question; e.g., $B-\phi-C-$ refers to a strain which is deficient in the synthesis of biotin, phenylalanine, and cystine. On the other hand, particular emphasis can be placed on the ability of a strain to synthesize a particular growth factor by suffixing a "+". Thus, $B-\phi-C-T+L+B_1+$

would refer to a strain deficient in the three factors mentioned above, but capable of growth in the absence of threonine, leucine, or of thiamine. The representation of a growth factor requirement by a minus sign is justified by the a priori consideration that a mutation establishing a growth factor requirement generally represents the loss of a function, and by the experimental finding of Beadle and Coonradt (1944) that wild are dominant to mutant genes in *Neurospora* heterocaryons. Strains which are "+" for all growth factors have been called prototrophs (Ryan and Lederberg, 1946), since this is the nutritional condition of the parental wild type *E. coli* strain from which all the mutants were ultimately derived.

In addition to these biochemical mutations, the character of resistance (abbreviated V_1^r) to coli phage T1 has been used. Spontaneous mutations for this character were selected by the method of Luria and Delbrück (1943) and are sufficiently rare (about 1 cell in 10^7 in a 24-hour culture from a small inoculum) to be negligible except when specifically selected for by applying excess phage to a large population of sensitive bacteria. There has been no indication in K-12 strains of any association between phage resistance and biochemical requirements, as reported by Anderson (1946) for mutants of another coli strain.

Several types of culture media were employed. The chemically defined minimal medium had the following composition, per liter: NH_4Cl, 5 g; NH_4NO_3, 1 g; Na_2SO_4, 2 g; K_2HPO_4, 3 g; KH_2PO_4, 1 g; $MgSO_4$, 0.1 g; glucose, 5 g; asparagine, 1.5 g; $CaCl_2$, trace; and trace element solution, 1 ml (as used for *Neurospora*; Beadle and Tatum, 1945). Agar was added at a concentration of 1.5 per cent when required, but to avoid flocculation the medium and the agar were autoclaved separately at twice the final concentration, and mixed before pouring plates. It was found that some lots of Difco agar were sufficiently free of the growth factors under consideration to make special washing unnecessary.

For nonsynthetic broths, "CC," consisting of peptone, yeast extract, and glucose, and "YB," the Difco product "yeast beef broth," were used.

One-ml inocula from broth cultures of distinct mutants (separately or in various combinations) were added to 50 ml YB in a 125-ml flask, and incubated for 24 to 48 hours at 30C with gentle shaking. After washing with sterile distilled water, samples of about 10^8 cells were inoculated into minimal agar pour plates, to which various supplements had been added as required. The plates were incubated for 48 hours and inspected for the presence of visible colonies. If these appeared, they were picked, suspended in sterile water, and tested for nutritional requirements. It was found that despite the heavy seeding of the plate, picking in this fashion ordinarily yielded homogeneous cultures, but for further study strains were subjected to serial single colony isolation on CC streak plates. Virus susceptibility was ascertained by cross-streaking the phage and the bacteria on a nutrient saline agar plate, as recommended by Demerec and Fano (1945), and recording whether lysis occurred at the intersection.

EXPERIMENTAL RESULTS

Spontaneous mutations of bacteria in pure culture were studied as a preliminary to the investigation of recombination. The over-all frequency of random biochemical mutations in untreated cultures is less than 0.1 per cent, although samples totaling not more than 5,000 cells have been studied so that the precision of this measurement is doubtful. In view of the low rate and random occurrence of such spontaneous mutations, however, they may be regarded as a negligible factor in this study.

The spontaneous reversion of biochemical mutants to prototrophs is under detailed investigation (Ryan and Lederberg) and will be reported on more fully elsewhere. It has been found that most biochemical mutants will revert at a low rate, prototrophs being found in the proportion of 10^{-7} in 24-hour cultures of single mutants. Reversions of different factors are, so far as has yet been ascertained, entirely independent; as predicted from the low rate of reversion of the individual factors, in ca. 10^{10} cells examined no instance was found in which reversion had occurred at both loci of a double mutant. Such a coincidence would have led to the appearance of a prototroph in a culture inoculated with a double mutant such as T−L−. On the basis of these considerations, only double and triple mutants have been used in the study of recombination.

The frequency of spontaneous mutations to virus resistance is of the same low order of magnitude as nutritional reversion. Mutations from resistance (V_1^r) to susceptibility (V_1^s) have not been described owing to the lack of efficient techniques for the detection of such reversions.

Prototroph recombination types. Since coincidental spontaneous reversion at two or more loci does not occur at a sufficiently high rate to be detected, the presence of prototrophs in mixed cultures of multiple mutants is evidence for gene recombination. Each mutant is capable of synthesizing all the growth factors for which it is not deficient; therefore, different mutants should have "+" alleles for all but the two or three mutant genes that characterize each strain. The segregation of prototrophic alleles of every gene into one cell would result in a prototrophic cell. It would develop into a visible colony on minimal medium, whereas other mutant cells would be unable to proliferate owing to the absence in minimal medium of their nutritional requirements.

When washed samples of mixed cultures of B−M−P+T+ and B+M+P−T− were plated into minimal medium, about 100 colonies developed for each billion (10^9) cells inoculated. No colonies appeared after inoculation of samples from the individual double mutants. One interpretation of the occurrence of prototrophs, designated as B+M+P+T+, is that the P+ and T+ genes of B−M−P+T+ and the B+ and M+ genes of B+M+P−T− have segregated into the same cell. This is a recombination hypothesis; alternatives will be discussed in the next section.

The possibility must be considered that the prototrophs consist of some sort of association of the unaltered mutants. In a classical illustration of nutritional symbiosis, or syntrophism (Lederberg, 1946), Valentine and Rivers (1927) showed that *Hemophilus canis* and *Hemophilus parainfluenzae*, which require X and V factor, respectively, would grow in mixed culture in media lacking these substances. They concluded that an exchange of these growth factors, synthesized by the bacteria, occurred through the medium. While there is no good reason to doubt this conclusion, these authors did not, in fact, conclusively demonstrate that this was the mechanism of the interaction. It is possible that cells were present in their mixed cultures which, as a result of gene recombination, required neither of the two factors. The situation is obscured by the use by these authors of serial transfers of large numbers of bacteria.

Syntrophism has been shown to occur with *E. coli* mutants (Lederberg, 1946). It is not likely, however, that it plays a significant role in the appearance of prototrophic colonies. Washed cells inoculated into minimal medium do not show syntrophism unless small quantities of their required growth factors are added. In minimal agar plates heavily inoculated with a washed mixed culture, a uniform turbidity does appear, which is ascribable to a limited exchange of factors and subsequent syntrophic growth.

Evidence of several sorts has been obtained for the homogeneity and uniqueness of prototrophs isolated from mixed cultures. Most significant, they are at least relatively stable, and attempts to detect mutant cells by an efficient selective technique (Lederberg and Tatum, 1946*a*, 1946*b*) have been unsuccessful. Massive doses of ultraviolet light, killing all but 10^{-6} of the cells in the culture, were no more successful in breaking up the supposed associations. In addition, prototrophs obtained from $B-M-P+T+V_1^r$ and $B+M+P-T-V_1^s$ were studied. Both susceptible and resistant cultures were obtained. Although one of the parental strains is resistant, the susceptible cultures were uniformly lysed upon application of the phage; on the other hand, there was no change in the nutritional behavior of cultures of resistant prototrophs subsequent to the application of the virus, which would be expected, in an association of the original mutants, to lyse the susceptible $B+M+P-T-V_1^s$ cells and leave only $B-M-P+T+V_1^r$. A nicotinicless mutant has been obtained by ultraviolet irradiation of a prototroph derived from $P-T-$ and $B-M-$. The prototroph in which this mutation occurred could have been neither a heterocaryon nor an association of diverse types, since in either case the absence of nic+ genes in the mutant would require the simultaneous mutation of more than one representative of this gene. This coincidence is highly improbable. The microscopic examination of seeded agar supported the conclusion that the cells of strain K-12 are well dispersed, so that most of the colonies that appear would be derived from single cells when only a few hundred cells are inoculated per plate, as was done after the initial isolation of prototrophs. Single cell isolations from a "recombination prototroph" strain have been made by Dr. M. Zelle of the National Institute of Health; all of the single cell cultures tested were of the same nutritional and virus-resistance type as the culture from which they were isolated.

Transformation. The evidence just presented points to the conclusion that the prototrophs are a new type of cell, which did not arise by spontaneous changes in a single double-mutant strain. Gene recombination, which was postulated above, is, however, not the only interpretation for the origin of these new types which would fit the evidence that has been presented. Alternatively, transforming principles, analogous to those demonstrated for pneumococcus serotypes, might participate in these genetic changes. The postulated transforming factors would be produced in one cell by genes in the "+" state, and after diffusion through the medium would convert mutant genes in another cell into their active, prototrophic alleles.

Since the conditions of the recombination experiments require that any transforming substance be present in the medium, an attempt was made to modify a nutritional mutant with a culture filtrate from another mutant. $B+M+P-T-V_1^s$ was grown in YB broth, and samples of 12- and 36-hour cultures were freed of cells by centrifugation and filtration through an ultra-fine sintered glass filter. The filtrate was diluted with an equal volume of YB and inoculated with $B-M-P+T+V_1^r$. As a control, $B-M-P+T+V_1^r$ cells were inoculated with $B+M+P-T-V_1^s$ into filtrate broth. After the cultures were incubated for 48 hours, they were analyzed for prototrophs by the methods described above. None were found in the $B-M-P+T+V_1^s$ cultures grown in the presence of $B+M+P-T-V_1^r$ filtrate, indicating the absence of an active transforming principle in the medium under these conditions. On the other hand, the growth in mixed culture of $B-M-P+T+V_1^s$ and $B+M+P-T-V_1^r$ cells resulted in the appearance of numerous prototrophs.

This failure to demonstrate a transforming principle cannot be regarded as rigorous proof of its absence, since there may have been subtle, undetected variations in the specific environmental conditions required for its activity. There is, however, further indirect evidence in a following section that such factors do not play an important role.

Combinations of other E. coli strains and conditions for recombination. Prototrophs have been obtained consistently in "crosses" of a variety of multiple mutants derived from K-12. There has been no exhaustive attempt to define the most favorable conditions for recombination. The best results have been obtained in rich, well-buffered media, cultured at 30C for at least 6 hours with moderate shaking. High salt concentrations, low and high temperature, diluted media, low buffering capacity, and low pH appear to be inimical to the recombination process even at levels which affect growth but slightly. Since *E. coli* strain B (Luria and Delbrück, 1943) has been used extensively in studies of bacterial mutation, an attempt was made to demonstrate the occurrence of a recombination process in this strain. Mutants were obtained which are summarized in table 1. Prototrophs were not found in mixed cultures of the double mutants of B/r. Similarly prototrophs were not found in mixtures of $A-M-P+T+$ (from B/r) and $A+M+P-T-$ (from K-12). There may be specific genetic requirements which must be fulfilled before recombination will occur.

Other recombination types. If prototrophs arise from the segregation into the same cell of + alleles of various genes, there should be present in the same cultures cells into which combinations of − alleles, different from those of the parental types, have segregated. In looking for these types, $B-\phi-C-T+L+B_1+V_1^s$ and $B+\phi+C+T-L-B_1-V_1^r$ were used, so that there were available 7 markers, some of which might be expected to segregate independently and give rise to new recombination types.

In order to detect these types, mixed cultures were plated into a minimal medium to which various supplements had been added, in different combinations.

In order to suppress the parental types, at least one of the requirements of each of the parental strains was withheld, and the supplements consisted of at most four factors, such as B, ϕ, T, B_1; B, ϕ, L, B_1; etc. An attempt was made at first to calculate the proportions of different recombination types simply from the number of colonies that appeared on different media. This method proved unsatisfactory with the markers used, since the variations in total numbers were so much greater than the differences found from one combination to another. Therefore, colonies were picked from various plates and suspended in water; growth after inoculation into a series of tubes containing all the supplements but one was used to classify the types. Unusual types were checked several times. For those plates so supplemented as to permit the development of colonies of a given type, the number of that type obtained was compared with the number of prototrophic colonies picked. This method permits a comparison of the relative numbers of prototrophs and of various other types. The results of such an analysis are summarised in table 2.

TABLE 2

Relative proportions of various nutritional cell types in a mixed culture of $B-\phi-C-T+L+B_1+V_1^s$ *and* $B+\phi+C+T-L-B_1-V_1^r$

TYPE	NUMBER OF THIS TYPE ISOLATED*	NUMBER OF PROTOTROPHS	RATIO OF THIS TYPE TO PROTOTROPHS	REMARKS
$B-\phi-C-T+L+B_1+V_1^s$	(Parental type. Present in large excess)			
$B+\phi+C+T-L-B_1-V_1^r$	(Parental type. Present in large excess)			
$B+\phi+C+T+L+B_1+$	86		1.00	Prototrophs
$B+\phi+C+T+L+B_1-$	36	37	0.97	Thiamineless
$B+\phi+C+T-L+B_1+$	2	31	0.06	Threonineless
$B+\phi+C+T+L-B_1+$	4	55	0.07	Leucineless
$B-\phi+C+T+L+B_1+$	5	56	0.09	Biotinless
$B+\phi-C+T+L+B_1+$	1	52	0.02	Phenylalanineless
$B+\phi+C-T+L+B_1+$	1	19	0.05	Cystineless
$B+\phi+C+T+L-B_1-$	3	16	0.19	Possible single-reversion type
$B-\phi-C+T+L+B_1+$	2	41	0.05	Possible single-reversion type
$B-\phi+C+T+L+B_1-$†	3	28	0.11	
$B-\phi+C+T-L+B_1+$†	(Isolated in a similar experiment)			
$B-\phi+C+T+L-B_1+$†	(Isolated in a similar experiment)			

* These figures do not include results of tests of virus resistance. Of 49 prototrophs tested, 20 (41%) were resistant. Seven out of 20 thiamineless (35%) were resistant.

† It should be noted that these types represent double-requirement recombination types.

In crosses between $B-\phi-C-T+L+B_1+$ and $B+\phi+C+T-L-B_1-$ the recombination types $B-\phi+C+T+L-B_1+$, $B-\phi+C+T-L+B_1-$, and $B-\phi+C+T+L+B_1-$ have been found. In addition, $B-\phi+C+P-T+$ was found in a mixture of $B-\phi-C-P+T+$ and $B+\phi+C+P-T-$. Furthermore, there have been numerous instances of recombination types consisting of a single biochemical requirement coupled with resistance or with susceptibility, when that same requirement in the parental type was coupled with the alternative response to the virus. To account for these recombinants, we postulate a sexual phase in this strain of *E. coli:* a cell fusion which allows the segregation of genes in new combinations into a single cell.

The alternative to this conclusion is that transforming substances, capable of inducing mutations *in both directions*, are present in the medium. No analogous system of transformations has been described. The possibility that spontaneous mutations for biochemical requirements occur in cells which are being transformed for other factors in one direction has been examined by looking for thiamineless ($B+\phi+C+T+L+B_1-$) mutants in a mixed culture of $B-\phi-C-T+L+B_1+$ and $B+\phi+C+T-L-B_1+$. None was found among 80 colonies which appeared on thiamine-supplemented agar, although they occur at a considerable rate in combinations of $B-\phi-C-T+L+B_1+$ and $B+\phi+C+T-L-B_1-$, as already pointed out. Bilateral transformations cannot be distinguished from a sexual process by the genetic analysis of a population. However, the relative frequencies of various types, as shown in table 2, provides indirect evidence on this point. In the cross between two triple mutants, the prototrophs are the most frequent type. This suggests that if transformation occurs at all, it is likely to affect all three "−" loci. A priori, one would expect the types in which only a single locus had been transformed to be the most frequent. These data on frequencies of various classes must, however, be interpreted with caution since selective differentials, which might operate even in a richly supplemented medium, have been neither completely controlled nor extensively studied.

DISCUSSION

A complete analogy cannot be drawn at present between the inheritance of bacterial characters and the Mendelian processes of higher forms. The occurrence in mixed cultures of bacterial mutants of new types, which can be represented as recombinations of the characteristics of the original strains, suggests very strongly that hybridization and segregation take place. However, the details of the genetic structure of these bacteria have not been ascertained as yet. We do not know how many alleles governing a particular character are present in each cell, how they divide in relation to cell division, nor their relationship to the nuclei which have been described in bacteria (Robinow, 1945). Further studies on the factors contributing to the relative proportions of different types may lead to the solution of these problems. Disparities in these proportions have already been mentioned; the apparent nonrandom segregation of phenotypic characters may be due to factor linkages, to cytoplasmic transmission, or to other processes. A possibly similar situation has been reported in interspecific hybrids of yeasts by Lindegren (1945). Although the isolation of ascospores should allow precise tetrad analysis, in most instances there was not a clear-cut segregation of vitamin requirements, but, instead, prototrophs predominated. This was ascribed to "a cytoplasmic mechanism . . . obscuring the Mendelian ratios."

The utilization of nutritional requirements in the study of recombination illustrates the principles of a more general method. Any mutation which can be selected for (or against) in competition with the parental types could be used. Comparable results should then be obtained with such characters as virus resistance, resistance to antibiotics and other chemical agents, sugar utilization, and agglutination with antibody or lysis in the presence of complement. Such reagents may extend the general approach of recombination study to organisms which are not well adapted to the application of nutritional methods.

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SUMMARY

Evidence has been presented for the occurrence of character recombination in the bacterium *Escherichia coli*. This suggests the existence of a sexual phase. Recombinations of genes controlling several growth factor requirements and resistance to a specific bacteriophage have been found.

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TURBIDIMETRIC EVALUATION OF BACTERIAL DISRUPTION BY SONIC ENERGY

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Marked interest has developed in the past few years in the application of sonic and ultrasonic energy to biological systems. The method to be described in this paper was developed for the purpose of evaluating the effects produced when bacteria are treated by intense sound. So often in the literature which has appeared regarding the action of sound on various systems, the only criterion of the energy applied has been a rather inexact description of power in terms of that utilized by the electrical system driving the transducer employed. But no consideration has been given to the relative efficiencies of the two systems, nor has a base line been established in terms of the energy actually being applied to the substances under treatment. Electromechanical systems have been evaluated in terms of either power input of the electrical system or its power output, failing to take into consideration such losses as may occur either in the electrical system itself or in the transducer system. This also applies to the losses which must obviously exist in the extremely high frequency equipments which employ a quartz crystal, and in which the coupling between the oil and the container for the material being subjected to sound energies is comparatively inefficient.

A series of experiments were undertaken to try to evaluate various vibrational devices in terms of the effects produced on organisms. These experiments were particularly timely in view of the fact that much interest has developed recently in the use of intense sound energies for the disruption of bacterial cells as a means of antigen production. Various experiments were conducted in which plate colony counts were employed as a measurement of the usefulness of the treatment of a viable suspension, or culture, of an organism. Attempts have been made to estimate the effects produced in terms of changes in protein nitrogen as a result of the rupture of bacterial cells. Both of these methods are laborious and time-consuming.

In the course of experiments conducted by the writer, one of the most noticeable effects was an increase in the apparent transparency of a suspension of organisms with the time of treatment. Consequently, a series of turbidimetric measurements were made and are reported herewith.

METHODS AND EQUIPMENT

Vibrational method. A combination comprising an electronically operated oscillator connected to a magnetostrictor transducer made by the Raytheon Manufacturing Company, Waltham, Massachusetts, was used for most of these experiments. This particular device had been developed as a laboratory unit for the treatment of small quantities of liquids at intense sound levels. The electrical portion of the equipment consists of an oscillator circuit, a power driver, and the associated power supply for the unit. Connected to it is a water-jacketed stainless steel cup, the bottom of which is a diaphragm actuated by a laminated nickel rod of square cross section, which, when properly polarized, alternately elongates and contracts in an alternating electromagnetic field. The capacity of the stainless steel cup is about 60 ml. For reasons which will be apparent later, quantities on the order of 20 ml were used in many of the experiments. The entire system is critically designed so that the electrical portion of the equipment must be accurately tuned, to match the mechanical resonance of the transducer.

Turbidimeter. In measuring the turbidity of the various cultures and suspensions which were used for the test, a photoelectric type of instrument was employed. The light source was a General Electric AH4 lamp operated from a special power transformer and with an optical system consisting of a condenser, slit, and collimator. Two barrier layer photronic cells in a null circuit were balanced by means of a high sensitivity suspension type galvanometer. Zeroing was accomplished by optically controlling the illumination falling on the reference cell. Material being measured was in a calibrated test tube placed before the working cell and balanced electrically against the potential provided by the reference cell. It was found that the organisms, the suspensions of which are reported here, had maximum absorption in the red end of the spectrum and that their concentration, when measured turbidimetrically with a wratten 25A filter, followed Beer's law. A sterile culture medium was used as a blank. In most cases the culture medium employed was Difco nutrient broth. Many organisms were tested, but only the following ones are reported: *Escherichia coli*, *Proteus vulgaris*, *Bacillus subtilis*, hemolytic streptococcus (Lancefield's group B), and *Neisseria gonorrhoeae*.

Technique of experiments. A quantity of a turbid culture of test organisms was placed in the transducer cup, which was cooled by tap water, and the materials were subjected to sonic vibration for fixed periods of time. In some experiments, the time of treatment and the quantity of liquid were constant, and the voltage, measured across the transducer, was varied. In other experiments, the frequency, voltage, and time were constant, and the quantity of liquid under treatment was varied. And in still others, the quantity, time, and voltages were constant, while the frequency was varied by a definite amount on either side of that of resonance in order to estimate the effect of dissonance.

Technique of turbidity measurements. The equipment was adjusted for zero by matching the test tube in front of the reference cell with a similar test tube placed in front of the working cell with the potentiometer scale indicating maximum resistance. Both of these tubes contained a sterile culture medium. The turbidity of the material before treatment was then determined, and progressive changes were measured during the course of these experiments. In all cases the same calibrated tube was used for measurement purposes in front of the working cell. Because of the fact that a balanced cells circuit was employed and that line voltage fluctuations equally affected both the working and reference cells, readings were reproducible and were considered to be quite accurate.

All absorptions are reported in terms of an absorption coefficient multiplied by a constant. Had cells been used with parallel faces that were optically flat, this constant would have been proportional to the length of the absorbing path. However, since test tubes were employed, the ordinates of the accompanying graphs represent the absorption coefficient multiplied by a constant, and this numerical value equals the difference between the natural logarithm of the transmission with no organisms in the suspending liquid, and the natural logarithm of the transmission of the liquid with organisms present.

It will be noted in all the curves presented herewith that with increased time of treatment there is a tendency for the curves to become asymptotic, but that the apparent position of this asymptote along the axis of absorption coefficients does not coincide with zero. A rather obvious explanation of this fact is that the residual absorption represents the fragments or "ghosts" of destroyed organisms. Checks made by colony counts have shown that the concentration of viable organisms under treatment will, in the case of *E. coli* for instance, decrease to less than 0.1 of 1 per cent of the initial concentration in less than 5 minutes' exposure to sonic energy. In other words, an initial concentration of approximately 10^8 organisms per ml will have been reduced to 10^5 organisms per ml after 5 minutes' vibration. Consequently, the error introduced by superimposing a concentration scale on the scale of absorption coefficients, as was done in figure 1, is extremely small.

EXPERIMENTAL WORK

E. coli: The effect of voltage. A unit quantity of a 21-hour culture of *E. coli*, strain B, obtained from the Johnson Foundation, University of Pennsylvania, was treated at approximately 9,000 cycles per second with the system adjusted to resonance and progressive decrease in turbidity measured at three voltage levels across the transducer. The results of this experiment are shown in figure 1.

Effect of power and time. From these data figure 2 is constructed showing the interrelationship of power (as a function of voltage), time, and concentration of a unit quantity of culture. A series of curves such as these could be of particular interest for selecting the proper time, or voltage, or power required in order to accomplish a specific effect in terms of concentration percentage.

Effect of variation of quantity. Figure 3 shows the decrease in effectiveness of treatment, in terms of the absorption coefficient of the suspension of organisms, with increase in the quantity of material being treated.

Figure 4 shows the decrease in turbidity of a 20-ml culture of *E. coli* in 4 minutes of treatment with a different transducer from that employed in the previous experiments.

Proteus vulgaris. Proteus vulgaris was selected as being an organism producing a fairly high degree of turbidity in a comparatively short time in a nutrient broth, and responding easily to rupture by intense sound energy. In this particular experiment, after many adjustments a frequency was selected in which the effectiveness of treatment, as measured by the progressive decrease of turbidity of the culture, was found to be at a maximum. The curve A in figure 5 shows the change in turbidity with respect to time at a frequency of 9,045 cycles.

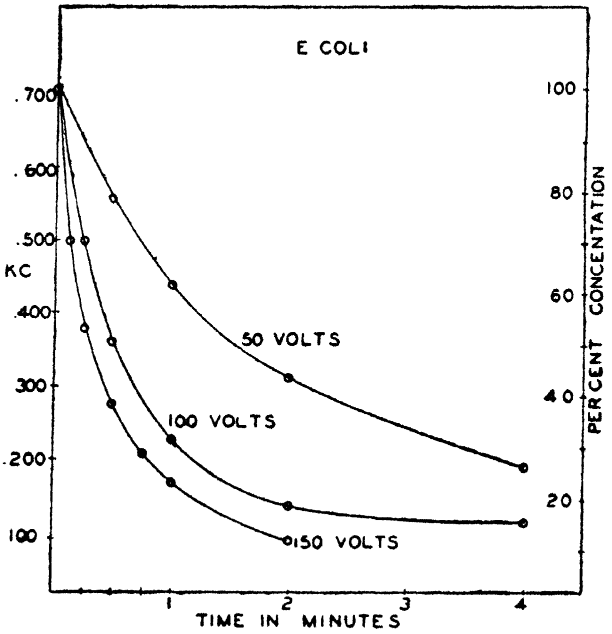

FIG. 1. DECREASE IN TURBIDITY OF E. COLI CULTURE WHEN TREATED SONICALLY AT DIFFERENT VOLTAGE LEVELS

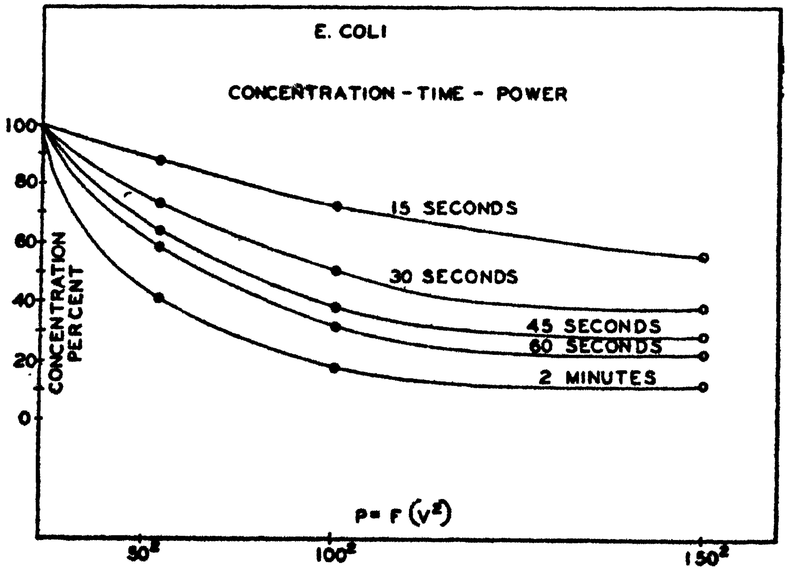

FIG. 2. RELATIONSHIP AMONG CONCENTRATION OF E. COLI CULTURE, TIME OF TREATMENT, AND POWER AS A FUNCTION OF VOLTAGE

This particular frequency is of no significance, in the opinion of the writer, as far as organisms themselves are concerned, and only represents that frequency at which the transducer system with its liquid load is most efficiently operated.

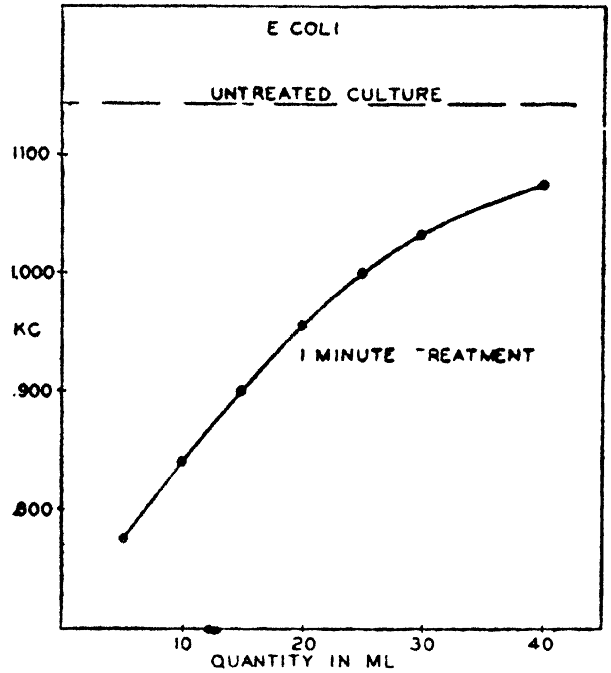

FIG. 3. RELATIONSHIP BETWEEN QUANTITY OF CULTURE UNDER TREATMENT AND EFFECTIVENESS OF BACTERIOCLASIS

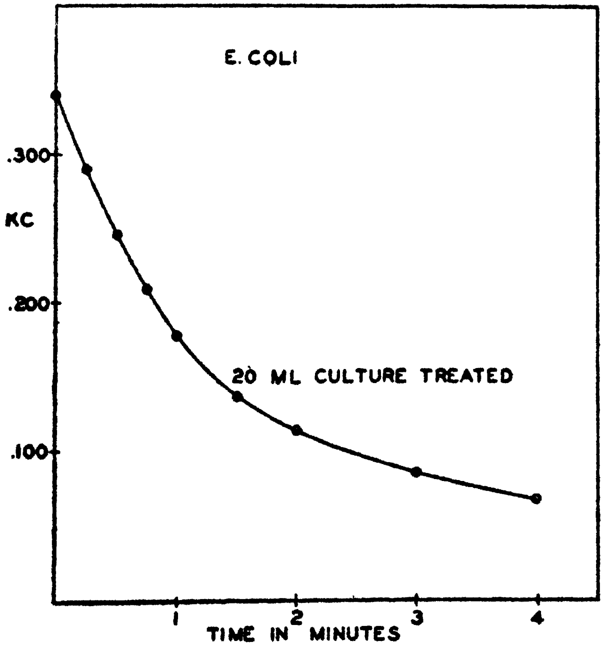

FIG. 4. DECREASE IN TURBIDITY OF E. COLI CULTURE WITH INCREASED TIME OF TREATMENT

It is a function only of the electrical and mechanical components of the system and is in no way associated with the dimensions of the organism or its species.

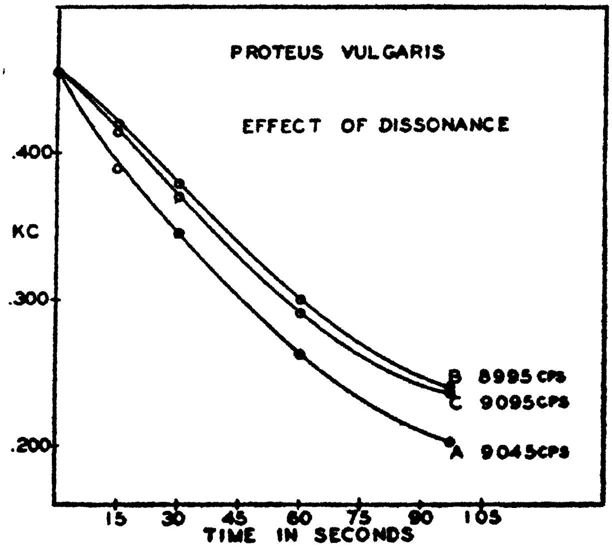

Fig. 5. Turbidity Changes When Proteus vulgaris Is Treated at Resonance and Dissonance

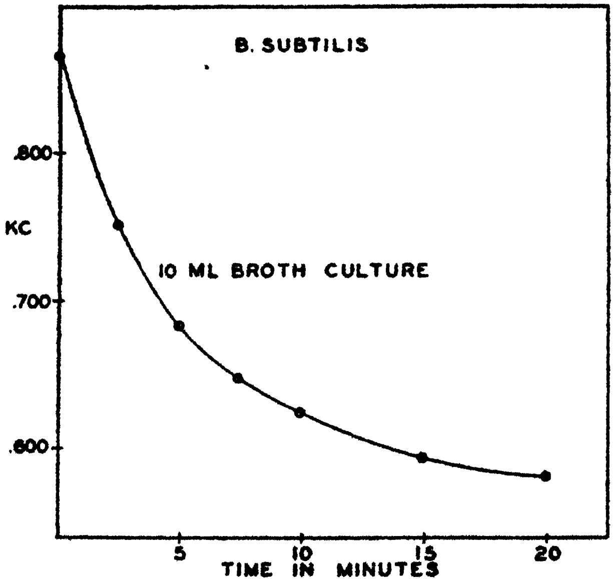

Fig. 6. Decrease in Turbidity When B. subtilis Culture Is Treated

However, in order to demonstrate the significance of dissonance in terms of the effectiveness of cell rupture, the frequency was altered by 50 cycles on either side of the selected optimum of 9,045 cycles. Curves B and C in figure 5 clearly demonstrate the significance of even so small a change as 50 cycles in the tuning of the system. It will be noted that curves B and C very nearly coincide, and from this one may assume that, for the particular condition of the experiment, 9,045 cycles was very nearly the most efficient frequency.

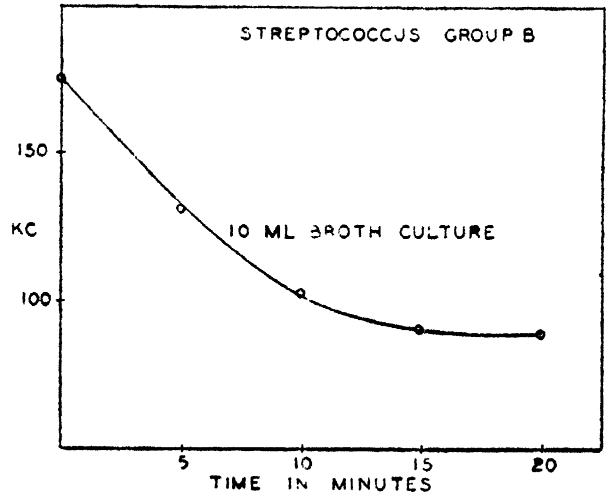

FIG. 7. CHANGES IN THE TURBIDITY OF A STREPTOCOCCUS CULTURE WITH TIME OF TREATMENT

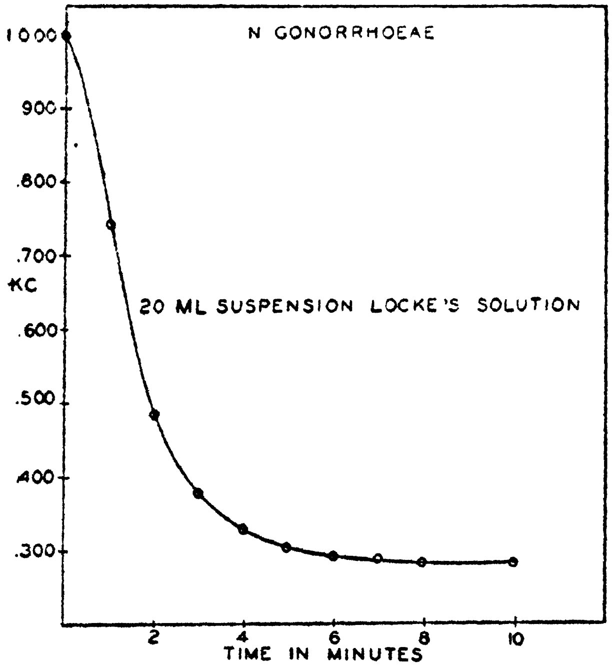

FIG. 8. DECREASE IN TURBIDITY OF N. GONORRHOEAE WITH INCREASED TIME OF TREATMENT

Bacillus subtilis. *Bacillus subtilis* was found to be one of many organisms comparatively resistant to destruction by means of sonic energy. Figure 6 shows the decrease in turbidity with time of a 10-ml culture of *Bacillus subtilis* treated for a total period of 20 minutes at constant voltage and at maximum effective frequency.

Hemolytic streptococcus. Figure 7 shows the decrease in turbidity of a culture of hemolytic streptococcus (Lancefield's group B) with time of treatment, at approximately 9,000 cycles per second. Ten ml of the culture were used. Constant voltage was maintained and the time of treatment was extended to 20 minutes.

Neisseria gonorrhoeae. A comparatively turbid suspension of *Neisseria gonorrhoeae* in Locke's solution was treated in the cup transducer for an extended period of time, and the decrease in turbidity measured at frequent intervals up to 10 minutes. In this case, Locke's solution was used as a blank rather than a sterile culture medium. The suspension was furnished to the laboratory by Dr. H. E. Stokinger of the University of Rochester, who subsequently reported to the writer that at the end of the treatment period no intact cells could be detected microscopically. Figure 8 shows the change in turbidity of this suspension with time of treatment.

DISCUSSION

The method of measuring the effectiveness of destruction of bacterial organisms by changes in turbidity of the solution under treatment would appear to be a simple one by which some base line of comparison between various vibrating devices can be established. Because of the difficulty of determining, or even estimating, the proportion of energy supplied by the electrical component of any particular system which is being transferred to the liquid under treatment, this method of evaluation, in terms of the effect produced, would seem to be sound. Furthermore, if the effect produced by a particular device is measurable, it becomes a means of gauging adjustment for most effective operation. Also, with the present widespread interest in the application of sonic, supersonic, and ultrasonic energy to various systems, there is a real need of some means by which these various systems in use can be compared with one another. By this technique the results obtained by one investigator with one type of apparatus may be compared with those of another, employing a different type of equipment.

In the past, attempts have been made to measure the effectiveness of breakup of bacterial cells by means of colony counts, which is undoubedly accurate when a comparatively few individuals are involved, but in the concentrations which are of interest to those experimenters who are concerned with the rupture of high concentrations of organisms, for the purpose of the extraction of their cell contents, either for production of antigens or for purposes of study, a simple rapid method with a reasonable degree of precision would seem most useful.

SUMMARY AND CONCLUSIONS

A method is submitted for the turbidimetric evaluation of bacterial disruption by sonic energy that seems to have a high degree of reproducibility.

This method appears sensitive to comparatively small changes in either the device which applies sonic energy to the liquid under treatment, or changes in the quantity of liquid or time of treatment of the material.

This method is independent of the instrument used for the measurement of turbidity, because of the fact that the findings are recorded in terms of the absorption coefficients multiplied by a constant.

TWO ANTIBIOTICS (LAVENDULIN AND ACTINORUBIN) PRODUCED BY ACTINOMYCES

I. Isolation and Characteristics of the Organisms[1]

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Two strains of *Actinomyces* showing antibacterial properties of special interest were isolated from greenhouse soil during a search for antibiotics effective against gram-negative and acid-fast microorganisms. One strain, designated as A-10, appears to be a variant of *Actinomyces lavendulae*. Although a similarity to streptothricin (Waksman, 1945) was noted in the spectrum obtained from limited antibacterial tests in which the unpurified product of A-10 was used, the culture differed so much in appearance, nutritional requirements, and other respects from *A. lavendulae*, a known producer of streptothricin, as to merit further study. More extensive antibacterial tests, performed later with the purified antibiotic, showed it to be distinct from streptothricin. We have named it *lavendulin*. The second strain of *Actinomyces* was found to produce an antibacterial substance resembling streptothricin and streptomycin but distinct from either of these. This strain of *Actinomyces*, designated as A-105, resembles in some respects *Actinomyces erythreus*, *A. fradii*, *A. albosporeus*, and *A. californicus* without being unmistakably one, or a variant of any, of these species. We have named the antibiotic produced by strain A-105 *actinorubin* because of the characteristic red mycelium which the organism forms on many media.

This paper describes the methods by which the strains of *Actinomyces* were isolated, their cultural characteristics, the cultural conditions which were observed to yield maximum production of the antibiotic substances, and their antibacterial properties *in vitro*. The methods for the chemical purification of lavendulin and actinorubin will be described elsewhere by Junowicz-Kocholaty and Kocholaty. The chemotherapeutic studies on lavendulin and actinorubin will be published by one of us (Morton, 1947).

Methods used in isolating from nature organisms which inhibit bacteria. The methods used for isolating and testing A-10, A-105, and A-82, the latter an organism producing an antismegmatis factor (Kelner and Morton, 1946), were similar. Our procedures were adapted from those described by Waksman, Bugie, and Schatz (1944) and in general resembled those described by Emerson *et al.* (1946). The actinomycetes were isolated from greenhouse soil (a rich and convenient source) by plating the soil in nutrient agar and selecting colonies

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[1] This project has been supported by the Smith, Kline, and French Laboratories, Philadelphia, Pennsylvania.

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of actinomycetes for isolation. The selection of colonies of actinomycetes was made entirely at random, no attempt being made to pick colonies differing from one another in appearance, nor to favor colonies which were inhibiting other soil organisms growing in the same plate. We assumed that different strains of the same species of *Actinomyces* differed in antibiotic potentialities and that colonies apparently inactive on the primary isolation plate might be active producers of antibiotics after prolonged incubation. Preliminary enrichment of the soil with dead or living bacteria was not found necessary, nor was the soil plated out on washed agar containing living bacteria as the sole nutrient. This confirmed the findings of Waksman and Schatz (1946), who showed such techniques to be of little value. The preliminary screening of the isolates for antibiotic activity was made by growing them on plain nutrient agar for 4 to 8 days at room temperature, after which their action against *Escherichia coli*, and sometimes *Mycobacterium smegmatis*, was determined by streaking the bacteria on the same plate and observing zones of inhibition.

Actinomycetes markedly inhibiting one or both bacteria were regrown, and tested this time against eight species, *Staphylococcus aureus*, *E. coli*, *Eberthella typhosa*, *M. smegmatis*, *Neisseria catarrhalis*, *Bacillus mycoides*, and *Bacillus subtilis*. This limited spectrum was selected as likely to indicate the possibility of previously unknown antibiotics. Actinomycetes that it seemed desirable to study further were grown in fluid media at 28 C. This was a criterion used in the screening process, for the many organisms which showed great activity on agar but produced little or no antibiotic in fluid media were considered to be of no practical value. The cultures were first grown in three representative fluid media—nutrient broth, nutrient broth plus glucose, and starch tryptone broth. For stationary cultures the media were made semisolid with 0.25 per cent agar. Isolates failing to form antagonistic substances in one of the media of at least 20 dilution units per ml against *Escherichia coli* or *Mycobacterium smegmatis* were discarded, i.e., a 1:20 dilution of the *Actinomyces* culture in agar failed to inhibit the growth of these two microorganisms. The spectrum of the more promising antagonists was redetermined, using the agar dilution assay method. The relative sensitivities of the eight bacteria were usually similar to those obtained in the first spectrum, but often marked differences appeared. By systematic variation of the best of the three media it was often, but not always, possible to increase the yield 2 to 20 times. No chemical purification of the antagonistic substance was attempted unless it was possible to obtain crude filtrates assaying at least 100 dilution units per ml of agar against *E. coli*. The majority of antagonists isolated yielded filtrates assaying no more than 20 or 40 dilution units, and unless the titer was easily raised, these were abandoned.

The cultural characteristics of the *Actinomyces* cultures, and such properties of the antibiotic as the detailed spectrum, stability to heat, acid, and alkali, relation of activity to pH, salts, etc., yielded additional evidence bearing on the novelty of a crude antibiotic. It was recognized, however, that definitive chemical analyses of the purified antibiotics was also necessary in determining their newness.

One of the tests used in evaluating a crude antibiotic was its toxicity when injected intraperitoneally into mice in 1- or 2-ml portions. It was realized that the amount of active substance present in the injected material might be too small to produce evidence of an inherent toxicity. Similarly, with an impure preparation an observed toxicity could not be attributed with certainty to the active principle. Nevertheless the experience of Fleming (1929), who demonstrated the low toxicity of penicillin before its chemical purification, indicated the usefulness of the test.

Mice tolerated sterile culture media such as the three afore-mentioned basic media used for actinomycetes, as well as glucose yeast-extract peptone broth, Czapek, and Raulin's medium (pH 4). Media containing corn steep were toxic. Filtrates of cultures of *Actinomyces antibioticus* (crude actinomycin) and *Penicillium flexuosum* (probably crude clavacin) killed mice. The great majority of the filtrates of strains of *Actinomyces*, which inhibited *E. coli* or *M. smegmatis* at a dilution of 1:20 or 1:40, did not kill mice. Toxic filtrates were not common. When a choice had to be made between antibiotics, both equally promising in other respects, the toxicity was the determining factor. Several cultures were discarded on this basis.

All assays at this stage were done by the agar dilution method, in Difco nutrient agar of final pH 7.3. The growth in the assay agar of some organisms, such as A-105 which developed at 37 C, was suppressed by adding crystal violet in the final concentration of 1:1,000,000 to the nutrient agar, which did not alter the end point but did facilitate reading of the plates. Crystal violet agar was also useful for the assaying of nonsterile filtrates from bacilli and aspergilli.

Cultural requirements of strain A-10. Strain A-10 cannot be grown for more than a few generations on glucose asparagine agar, a medium on which a streptothricin-producing *A. lavendulae*, which we have examined, grows well. On a richer medium, such as nutrient agar, which supported its growth, sporulation of A-10 was poor. A search was made, therefore, for a medium rich enough to permit growth and to stimulate sporulation of *Actinomyces* A-10. Tryptone starch agar fulfilled these requirements; it was further enriched without sacrifice of its sporulation-stimulating properties. The final medium (M-130) contained 5 g tryptone, 10 g starch, 3 g Difco beef extract, 2 g glucose, 1.2 g Na_2HPO_4, 0.8 g KH_2PO_4, 0.1 g $FeSO_4 \cdot 7H_2O$, and 15 g agar. The ingredients were dissolved in 1,000 ml potato extract made by simmering 400 g white potato slices for 40 minutes in 1,000 ml water (distilled water, or preferably, spring water). The decanted liquid is used.[3] Not only A-10 but most of the 200 strains of *Actinomyces* isolated during the investigation grew and sporulated well on this medium.

Media for the production of lavendulin. Titers of 200 to 400 dilution units against *E. coli* were formed by A-10 when grown in stationary culture at 28 C on a medium containing 5 g Difco peptone, 3 g Difco beef extract, 10 g glucose, 20 ml molasses ("Brer Rabbit," green label), 2.5 g agar, and 1,000 ml distilled water; pH 6.0. The peptone could not be replaced by tryptone without reduction in the yield of antibiotic. A detailed study of nutritional requirements for stationary cultures was not made. The maximum titer is reached about the sixth day, at which time the pH is about 5.6. The medium at this time is covered by a thick, gray, partially submerged pellicle.

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[3] In at least two cases potato extract was also useful as a substitute for corn steep in media for the production of antibiotics. Either fresh potato extract or corn steep stimulated antibiotic production by a *Bacillus* (H-40), and either fresh or dried potato extract (kindly prepared for us by the Difco Laboratories) or corn steep stimulated antibiotic production by an *Actinomyces* (R-13).

In shake cultures a study was made of the effect of beef extract on the yield of antibiotic. Table 1 shows that in the presence of beef extract the yield is reduced; the medium also becomes much more acid, suggesting that growth factors or salts contained in the beef extract affect the type of carbohydrate metabolism.

TABLE 1

Effect of varying the composition of the culture medium on the production of lavendulin

MEDIUM	DAYS	2	3	4
M-120*	pH† Activity‡	4.5 40	4.5 20	4.2
M-10.6§	pH Activity	8.2 200	8.2 100	8.2 40
M-10.0‖	pH Activity	5.8 200	7.4 200	7.8 200 (400)
M-10.5¶	pH Activity	7.4 400 (1,000)	7.4 400 (1,000)	7.4 1,000

* M-120—5 g Difco peptone, 3 g Difco beef extract, and 10 g glucose.

† The pH of the culture at the time of assay.

‡ Activity expressed in dilution units per ml of agar against *E. coli*. The figures in parentheses indicate almost complete inhibition of *E. coli* at the dilution indicated.

§ M-10.6—M-120 without beef extract.

‖ M-10.0—5 g Difco peptone, 3 g Difco beef extract, 10 g glucose, and 20 ml molasses.

¶ M-10.5—M-10.0 without beef extract.

Our best medium for the production of antibiotic is one composed of 5 g Difco peptone, 10 g glucose, and 20 ml molasses in 1,000 ml distilled water. Titers of 1,000 dilution units against *E. coli* have been obtained consistently.

Cultural characteristics of Actinomyces, strain A-105. Strain A-105 is characterized by ability to grow well on Czapek agar, by lack of soluble pigment, by failure to blacken peptone media; by a yellowish to intense red vegetative mycelium and by white to pink aerial mycelium. Oval conidia are borne in chains, and spirals are abundant in many media. Optimum temperature for growth is 37 C; very poor growth was obtained at 15 C. Action in milk is variable; gelatin is liquefied. On nutrient agar containing 3 to 5 per cent glycerol, the mycelium was yellow to an intense fuchsinlike red color, especially on first isolation, and with no spores. The red mycelium is characteristic on calcium malate agar, on Czapek agar, and also on most media in the presence of a *Bacillus* contaminant.

Media for the production of actinorubin. Crude actinorubin for chemical study was produced by surface cultivation at 28 to 30 C on a medium composed of 5 g Difco tryptone, 1.2 g Na_2HPO_4, 0.8 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 10 g white sugar (commercial or purified sucrose), 2.5 g agar, and 1,000 ml distilled water; final pH was 6.8 to 7.2.

In surface culture, tryptone could be replaced, with somewhat reduced yields, by asparagine, "phytone," or dehydrated skim milk, but not by Difco peptone, neopeptone, nitrates, or gelatin. In the presence of Difco peptone, the medium became alkaline very much more quickly than with tryptone. This increase in alkalinity may be indirectly the cause of reduced antibiotic production. White sugar (sucrose) could be replaced by brown sugar, molasses, or lactose with an equal or a somewhat reduced yield of antibiotic. Starch and glycerol were unsatisfactory.

Unless iron was added to the medium, antibiotic production was very low, although growth was relatively unaffected As little as 5 mg $FeSO_4 \cdot 7H_2O$ per liter of medium gave good yields of the antibiotic. Use of 20 mg $FeSO_4 \cdot 7H_2O$ decreased the yield slightly and caused the pH of the medium to fall to 5.6 to 6.0 within 7 days, as compared to pH 7.0 to 7.4 for media containing 10 mg $FeSO_4 \cdot 7H_2O$. Slight changes in the concentration of $MgSO_4$ and KCl had little effect.

Little or no antibiotic was produced in the white sugar tryptone medium (agar omitted) in shake culture, apparently because of very poor growth. Good yields were obtained, however, if 10 ml molasses ("Brer Rabbit," green label) per liter were substituted for the white sugar. Equal or somewhat reduced yields were also obtained if brown sugar, lactose, or starch was substituted. There was no antibiotic produced in plain nutrient broth. In a molasses tryptone medium containing only 25 per cent as much phosphate as usual, the yield was reduced by about one-half. The importance of the carbohydrate-nitrogen balance is indicated by rapid alkalinization and reduced yield when 10 instead of 5 g tryptone per liter of medium were used.

In surface cultivation on the white sugar tryptone medium, 200 to 400 dilution units per ml against *E. coli* were obtained in 8 to 10 days. The pH tended to rise to 7.2 to 7.5 in the first 3 to 5 days, slowly drop to 6.6 to 6.8 at about the tenth day, then slowly rise during the next week to about 7.8. In brown sugar and molasses media, the maximum production of antibiotic was reached in 5 to 6 days. The white sugar medium was preferred because of its greater simplicity and because the maximum titer, once reached, persisted longer.

Table 2 shows two experiments in shake culture in molasses tryptone medium at a temperature of 28 C, and illustrates the variation encountered. Yellow to salmon-colored, moderately coarse pellets were produced in shake culture in the molasses tryptone medium.

Bacterial spectrum of lavendulin and actinorubin. Partially purified preparations of the two antibiotics (Junowicz-Kocholaty and Kocholaty, to be published) were used for a comprehensive study of the action of lavendulin and actinorubin on a variety of bacterial cultures. Serial twofold dilutions of the active substances were made in Difco nutrient broth adjusted so as to have a reaction of pH 7.3 after sterilization. One loopful of the culture under test was inoculated into each dilution tube and a control tube of the culture medium. Incubation of the tests was at 37 C for 18 to 20 hours. The antibiotics were standardized against *E. coli*, strain P216. The smallest amount of the antibiotic per ml of Bacto-nutrient broth, pH 7.3, which prevented growth of *E. coli* under the conditions of the test was designated as one dilution unit. The results of testing the two antibiotics against many strains of microorganisms are summarized in table 3.

From the data listed in table 3 it appears that, in general, actinorubin and lavendulin are similar in their antibacterial action against most strains of microorganisms. However, the differences in the susceptibilities of *Corynebacterium diphtheriae*, P1, *Micrococcus aurantiacus*, P103, and *Sarcina lutea*, P6, to the two antibiotics were greater than the anticipated experimental error.

At the time our tests were being conducted, Price, Nielsen, and Welch (1946) reported on the sensitivity of *Bacillus circulans* to streptomycin. We obtained a subculture of this strain of *B. circulans* from these authors and tested its sensitivity to streptomycin sulfate, lavendulin, and actinorubin in Difco nutrient broth, pH 7.3, by our technique of assaying. The sample of streptomycin sulfate was assayed against the strain of *E. coli* employed for standardization of lavendulin and actinorubin and under the same conditions employed for assaying these antibiotics. *B. circulans* was 32 times more resistant to actinorubin and 2.5 times more resistant to lavendulin than to streptomycin. Another member of the genus *Bacillus*, *B. megatherium*, P97, was tested against the three antibiotics under identical conditions. It was 16 times more resistant to actinorubin than to streptomycin. Its resistance to lavendulin and streptomycin was of the same order. From the results obtained by testing *B. circulans* and *B. megatherium* against streptomycin and actinorubin it appears that the two antibiotics are different.

TABLE 2

Production of actinorubin in shake culture with tryptone molasses medium

DAYS	1	2	3	4	5	6	7	8	9	16
pH*		7.0	7.0	7.2	7.8	8.0			8.0	
Activity†		400	400(1,000)	400(1,000)		200(400)			200(400)	
pH	6.8	7.0	7.4	7.8	7.8		7.8	8.0	7.8	
Activity	10	200(400)	100(200)	100	40(100)		40(100)	40	40	10(20)

* The pH of the culture at the time of assay.

† The dilution units per ml of agar determined against *E. coli* equal the inhibiting dilution. The figures in parentheses indicate almost complete inhibition of *E. coli* at the dilution indicated.

In a personal communication from an investigator (Dr. E. J. Pulaski) working in another laboratory with many of our cultures, it was stated that *Chromobacterium violaceum*, P104, and *Serratia marcescens*, P4, were equally susceptible to streptomycin. The differences in susceptibility of these two cultures to actinorubin and lavendulin (table 3) suggest that both actinorubin and lavendulin are different from streptomycin.

TABLE 3

Bacterial spectrum of actinorubin and lavendulin

ORGANISM	STRAIN	NUMBER OF DILUTION UNITS/ML INHIBITING TEST ORGANISM*	
		Actinorubin	Lavendulin
Aerobacter aerogenes	P41	0.25	0.5
Alcaligenes faecalis	P61	2	4
Bacillus anthracis	P60	4	4
Bacillus anthracis	P119	2	8
Bacillus cereus	P109	32	64
Bacillus mesentericus	P112	0.5	0.5
Bacillus mycoides	P156	0.25	0.06
Bacillus mycoides	P33A	8	8
Bacillus mycoides, Waksman	P31A	16	32
Bacillus subtilis	P7	0.01	0.03
Bacillus subtilis, Merck	P23A	0.5	0.125
Bacillus subtilis, Koch	P219	8	16
Brucella abortus†	P18A, P62	4	16
Brucella melitensis†	P80	8	8
Brucella suis†	P64	16	32
Chromobacterium violaceum	P104	0.015	0.025
Corynebacterium diphtheriae	P1	0.015	4
Corynebacterium xerose	P83	0.004	0.007
Diplococcus pneumoniae‡	P27, P29	>128	128
Eberthella typhosa	P115	0.25	0.015
Eberthella typhosa, nonmotile	P11	0.5	1
Escherichia coli	P216	1	1
Escherichia communior	P218	0.5	1
Gaffkya tetragenae	P43	0.03	0.015
Klebsiella pneumoniae	P163A	0.125	0.03
Micrococcus aurantiacus	P103	0.007	0.000007
Mycobacterium smegmatis	P49	1	1
Mycobacterium tuberculosis, bovis§	Ravenel	8	6
Neisseria catarrhalis	P66	0.03	0.06
Proteus vulgaris	P98	1	2
Pseudomonas aeruginosa	P186A	32	128
Salmonella enteritidis	P51	0.25	0.25
Salmonella paratyphi	P198A	0.25	0.06
Salmonella schottmuelleri	P35	0.125	0.125
Sarcina lutea	P6	0.007	0.5
Serratia marcescens	P4	4	4
Shigella dysenteriae	P52A	1	2
Shigella paradysenteriae, Flexner	P21	1	2
Staphylococcus aureus	P210	0.06	0.015
Streptococcus pyogenes‡	P24	128	128
Streptococcus, alpha‡	P169	>128	>128
Streptococcus, gamma‡	P26	128	128
Trichophyton interdigitale		8	16
Vibrio comma	P215	0.5	0.25

* One dilution unit is the smallest amount of the antibiotic per ml of Difco nutrient broth, pH 7.3, which prevents growth of *E. coli* under the conditions of the test as described in the text.

† Difco tryptose broth. Incubation period, 48 hours.

‡ Difco nutrient broth, pH 7.3, to which were added 0.5 per cent NaCl and 5 per cent sterile, defibrinated, normal horse blood.

§ Test made using the medium described by Crumb (1946) and the technique described by Wells (1946).

In some earlier work we tested streptothricin and a streptothricinlike compound for their antibacterial spectra. It was found that *Corynebacterium xerose* was about as resistant as *E. coli* to streptothricin. The great susceptibility of this strain to actinorubin and lavendulin suggests that these two antibiotics are different from streptothricin.

Development of tolerance of E. coli to actinorubin, lavendulin, streptothricin, and streptomycin. *E. coli*, P216, was inhibited by 0.06 units of streptomycin per ml of Difco nutrient broth, pH 7.3, for 48 hours at 37 C. At 48-hour intervals a new series of tubes of nutrient broth containing serially decreasing amounts of streptomycin was inoculated with 0.1-ml amounts of the culture growing in the maximum amount of streptomycin in the previous series of tubes. In the second and fourth series there was a gradual increase in the resistance of *E. coli* to streptomycin, each series of cultures being about twice as resistant as the previous series. The fifth serial transfer of the culture in the presence of streptomycin showed an abrupt change in tolerance for the drug, there being about a 16-fold increase. The organisms in the eighth series were over 100 times more resistant than those in the fifth series. In the twelfth serial transfer *E. coli* grew nearly as well in the presence of 1,600 units of streptomycin per ml of nutrient broth as in the nutrient broth without streptomycin. This represented a more than 26,600-fold increase in resistance of the culture to streptomycin. When this streptomycin-fast strain of *E. coli* was tested against lavendulin, actinorubin, and streptothricin, it was found to be nearly as susceptible to the three drugs as was the normal culture of *E. coli*. In the case of streptothricin, growth of the streptomycin-fast strain took place one tube earlier in the series than did the normal strain, whereas in the case of lavendulin and actinorubin growth took place two tubes earlier in the series than did the normal culture. Although these results represent a 2-fold or 4-fold increase in resistance, they are within the range of experimental error. When growth of the streptomycin-fast strain occurred in any of the tubes, it was similar to that produced by the normal strain of *E. coli*. In these results, also, streptomycin differed from streptothricin, actinorubin, and lavendulin.

During 10 serial transfers of *E. coli*, P216, in Difco nutrient broth (pH 7.3) containing actinorubin by a technique described above for streptomycin, the culture developed a 64-fold increase in resistance to actinorubin. When this actinorubin-fast strain was tested against streptomycin, streptothricin, and lavendulin, it appeared to be slightly more resistant to all three compounds. The actinorubin-fast strain was 16 times more resistant to streptomycin and 32 times more resistant to streptothricin and lavendulin. Growth of the actinorubin-fast strain was about one-half as vigorous as that of the normal strain.

During 10 serial transfers of *E. coli*, P216, in Difco nutrient broth (pH 7.3) containing lavendulin by a technique described above for streptomycin, the culture developed a 32-fold increase in resistance to lavendulin. When this lavendulin-fast strain was tested against streptomycin, streptothricin, lavendulin, and actinorubin, it was found that the increase in resistance to each of the four antibiotics was of the same order. Growth of the lavendulin-fast strain was about one-half as vigorous as that of the normal strain.

During 10 serial transfers of *E. coli*, P216, in streptothricin (medium and technique as above), the culture developed only an 8-fold increase in resistance to the antibiotic. When the streptothricin-fast strain was tested against streptomycin, actinorubin, lavendulin, and streptothricin, it was found that the increase in resistance to each of the four antibiotics was of the same order. Growth of the streptothricin-fast strain was about one-half as vigorous as that of the normal strain.

From these studies on drug fastness of *E. coli* to streptomycin, streptothricin, actinorubin, and lavendulin, it is readily apparent that actinorubin and lavendulin are distinct from streptomycin. Differences between actinorubin, lavendulin, and streptothricin were not readily detected by this method of testing. The usefulness of this method of testing antibiotics was pointed out by Eisman, Marsh, and Mayer (1946).

Effect of NaCl and blood upon the activity of actinorubin and lavendulin. Antibacterial tests were made in Difco nutrient broth, pH 7.3, employing twofold dilutions of the antibiotics and *E. coli* as the test organism, as described previously. When NaCl was added to the medium to the concentration of 0.8 per cent, 128 times more actinorubin and 256 times more lavendulin were required to inhibit the test organism as compared to the amounts of the antibiotics required to inhibit the test organism in the absence of added NaCl. The addition of 10 per cent sterile, defibrinated horse blood to the Difco nutrient broth containing 0.8 per cent NaCl did not alter the activity of the antibiotics.

Preliminary tests of the action of actinorubin and lavendulin in mice were promising (Kelner, Kocholaty, Junowicz-Kocholaty, and Morton, 1946) and are being reported elsewhere (Morton, 1947).

SUMMARY

Two strains of *Actinomyces* have been isolated which produce substances that appear to be different from previously described antibiotics. One of these new substances has been named *actinorubin*, the other *lavendulin*.

A description of the two strains of *Actinomyces* is given as well as conditions of growth for maximum production of the antibiotic substances.

The bacterial spectrum of actinorubin and lavendulin is given. The two substances appear to be different from each other and from streptomycin and streptothricin.

Much greater resistance of *Escherichia coli* to streptomycin could be developed than resistance to actinorubin or lavendulin.

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THE ACTION OF PASTEURELLA PESTIS BACTERIOPHAGE ON STRAINS OF PASTEURELLA, SALMONELLA, AND SHIGELLA

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Bacteriophages active against *Pasteurella pestis* have been described by a number of workers, including Flu (1927), Sugino (1932), Advier (1933), and Girard (1942). The subject has been reviewed by Harvey (1933) and Wu *et al.* (1936). This report describes certain relationships of *P. pestis* to *Pasteurella pseudotuberculosis* and to *Salmonella* and *Shigella* strains, as shown by bacteriophage action and confirmed to a limited extent by agglutination tests.

BACTERIOPHAGE EXPERIMENTS

Materials. The strain of phage used in this study was obtained from the Pasteur Institute of Dakar, Senegal, in 1945. It was originally isolated from a positive blood culture prepared from a clinical case of bubonic plague (Advier, 1933). The phage was passed several times in contact with liquid and solid cultures of *P. pestis*, avirulent strain A1122 (Jawetz and Meyer, 1943), using single plaque procedures as well as lysis in broth. A batch of phage, sufficient for the present study, was prepared in a meat infusion broth. This phage will be referred to throughout this paper as the "parent strain." At a later date, to rule out the possibility of contaminants, the phage was further purified by picking isolated plaques to broth and incubating in the presence of *P. pestis* A1122. This procedure was repeated six times. The resulting phage was considered to be free from any contaminants and is referred to below as the "purified phage."

The standard medium used was Difco proteose no. 3 agar. A liquid medium was prepared using the same formula without agar.

Methods. Standard agar plates, dried for several days at 37 C, were spread with from 0.1 to 0.25 ml of a young broth culture of the strain under investigation. The plates were held at room temperature until the liquid was completely absorbed, usually 5 to 10 minutes. The phage was then applied to marked areas, using a standard platinum loop having an inside diameter of 3.0 mm. Care was taken to avoid excessive disturbance of the film of organisms on the surface of the agar. It was possible to apply up to nine such phage areas on a single plate. The plates were incubated at 37 C after the phage was absorbed sufficiently to permit inversion.

All cultures found susceptible to lysis by the parent strain of phage, or by any phage adapted from the parent strain, were tested for lysogenic ability. The methods of Fisk (1942) were used for unmasking lysogenic strains. Except as otherwise noted, no evidence of lysogenesis was found.

Results

P. pestis. Twelve strains of human and animal origin were studied. Ten of these strains were avirulent for animals, two were fully virulent. All strains tested were susceptible to the parent strain of phage. Advier (1933), using the same phage, found that all of 47 strains of *P. pestis* were lysed; therefore no effort was made to test additional cultures. The properties of the parent phage were as follows:

On agar plates, a phage dilution of 10^{-6} gave an area of confluent lysis with all strains after 24 hours at 37 C. Higher dilutions, up to 10^{-8}, showed isolated plaques. All strains were similar in susceptibility, the only variations being within the limits of experimental procedures.

In liquid media, all strains were lysed in contact with a 10^{-8} dilution of the parent phage after 24 hours at 37 C.

On solid media, isolated plaques were about 3 mm in diameter in 24 hours. Plaques continued to enlarge with prolonged incubation at 37 C, or at room temperature, until the entire plate might be cleared.

No secondary growth was observed in liquid or solid media after lysis of normal strains of *P. pestis.* Lysates were sterile on subculture. On three occasions phage-resistant strains were obtained by passing cultures daily in broth for a number of transfers and then exposing the organisms to undiluted phage on agar plates. The phage-resistant strains showed no envelope substance microscopically, formed tiny (0.1 mm in diameter) colonies, produced a very granular growth in broth, and died out in a few months on storage at +4 C on blood agar slants. Advier (1933) reported no secondary growth following lysis of 47 strains of *P. pestis* using the same phage.

The thermal death range of the parent strain of phage was 61 to 63 C in 30 minutes.

The minimum time required for lysis was 40 minutes, when a broth culture was used showing turbidity barely visible to the eye (approximately 5×10^7 bacteria per ml) and when undiluted phage was added in an amount equal to 2 per cent of the volume of the culture. Older cultures and smaller amounts of phage prolonged the time required for lysis. On agar plates, plaques were visible within 2 hours after the addition of phage to a plate spread with 0.15 ml of a 24-hour culture of *P. pestis.*

The stability of the parent phage was marked. No appreciable loss in titer against the homologous strain of *P. pestis* was observed after 2 months at room temperature. Dilutions prepared in broth and stored at +10 C showed no loss in potency after 2 months.

P. pseudotuberculosis. Sugino (1932) reports absence of lytic action of *P. pestis* phage in contact with *P. pseudotuberculosis.* Advier (1933) made similar observations on three strains of *P. pseudotuberculosis,* using the same *P. pestis* phage employed in the present study. On the other hand, Girard (1942) reports a *P. pestis* phage able to lyse some strains of *P. pseudotuberculosis* to the same titer as *P. pseudotuberculosis* phage.

In the present study, 27 strains of *P. pseudotuberculosis*, at least 3 of which were from human cases, were investigated for sensitivity to the parent and purified *P. pestis* phages. All cultures were further examined for susceptibility to an adapted phage produced from a single plaque. The plaque was picked from a plate spread with *P. pseudotuberculosis* (Spokane strain) to which had been added undiluted *P. pestis* phage of the parent strain. After 12 passages in contact with broth cultures of *P. pseudotuberculosis* (Spokane), this adapted phage showed a definite increase in ability to lyse most strains of *P. pseudotuberculosis*, without loss of action on *P. pestis*. A comparison of results is shown in table 1.

TABLE 1

Lysis of P. pseudotuberculosis strains by phage

(Highest dilution showing confluent lysis on plates)

P. PSEUDOTUBERCULOSIS STRAIN	PURIFIED P. PESTIS PHAGE	P. PESTIS PHAGE ADAPTED TO P. PSEUDOTUBERCULOSIS
NIH 8-295	10^{-1}	10^{-8}
NIH K-1	10^{-3} R	10^{-5}
NIH B-111	10^{-4}	10^{-6}
NIH B-519	10^{-4}	10^{-6}
NIH 274	—	10^{-8}
Berk. C1903	10^{-4}	10^{-6}
Berk. D1774	—	10^{-5}
Berk. IRL	10^{-2} R	10^{-4} R
NCTC 1102	10^{-4}	10^{-6}
NCTC 2200	±R	10^{-6}
NCTC 2476	10^{-1} R	10^{-6}
NCTC 3570	10^{-3} R	10^{-5}
Jawetz 4	—	10^{-1}
Jawetz 5	10^{-1} R	10^{-1} R
S-274	10^{-1} R	10^{-2}
S-275	—	10^{-3}
K. Meyer 1	±R	10^{-6}
Seattle	—	10^{-2}
Spokane	—	10^{-3} R
Hobmaier	10^{-3} R	10^{-6}
New Orleans	—	10^{-5}
San Francisco	10^{-1}	10^{-1}
C. T. Snyder	—	10^{-3} R
Saranac	10^{0} R	10^{-5}
Moss	10^{-5}	10^{-5}
Mason	10^{-1} R	10^{-1} R
Topping	±R	10^{-4}
P. pestis A1122	10^{-6}	10^{-6}

± = isolated plaques, without confluent lysis, using undiluted phage.
— = no lysis with undiluted phage.
R = resistant growth.

The phage adapted to *P. pseudotuberculosis* showed the same general properties as the parent strain of *P. pestis* phage, except that an occasional small plaque, about 1 mm in diameter, was noted. It was felt that this discrepancy in plaque size might have indicated a lysogenic strain, but no evidence of lysogenic cultures could be obtained by the use of the methods of Fisk (1942) for unmasking lysogenic strains. It seems reasonable to assume that the results in table 1 were due to adaptation and not to the presence of contaminating phages.

The marked variation in susceptibility of different strains of *P. pseudotuberculosis* to the two phages used is of considerable interest. This point is discussed below.

It was hoped that the differences shown in table 1 might supply a means to differentiate *P. pestis* from *P. pseudotuberculosis*, a problem not always readily solved by cultural methods. Although such a differentiation is possible in most cases, it is felt that the observed reactions are not sufficiently reliable and reproducible to constitute a means of clearly distinguishing between the two organisms. Further studies are being conducted in an effort to establish a reliable method for differentiation.

It was of interest to note that most strains of *P. pseudotuberculosis* showed a definite increase or decrease in sensitivity to phage after prolonged storage on agar slants under oil. The relationship of this altered sensitivity to changes in antigenic structure will be studied further, in an effort to determine whether the sensitivity to *P. pestis* phage might be a quantitative means to measure antigenic relationships between *P. pestis* and *P. pseudotuberculosis*.

P. septica. Eleven strains of *P. septica* isolated from domestic fowl and one strain isolated from a gray squirrel were tested by the plate method for sensitivity to both the parent phage and the phage adapted to *P. pseudotuberculosis*. No evidence of lysis was observed. Five of these 12 cultures carried phage acting on themselves.

Salmonella. Forty-two strains of *Salmonella*, representing 25 species, were tested by the plate method against the parent strain of *P. pestis* phage. Three strains showed lysis, namely, *S. schottmuelleri* (no. 6), *S. hirschfeldii* (58G, no. 31), and *S. rubislaw* (phase I, no. 159). These three strains were lysed only by the undiluted phage, and secondary growth occurred on further incubation. In broth cultures, the addition of phage resulted in partial lysis with resistant growth. Repeated attempts to transfer the phage by serial subculture in broth were unsuccessful.

The 3 strains of *Salmonella* lysed by the parent phage, as well as many of those not lysed, had been maintained on agar for several years. The 3 strains susceptible to the phage were predominantly in the rough phase. At least 12 of the strains that were not affected by the parent strain of *P. pestis* phage were also in the rough phase. Cultures which could not be lysed included 3 strains of *S. schottmuelleri* and 2 of *S. hirschfeldii*. The phage action, therefore, was not related to the age, the species, nor the state of dissociation of the cultures studied.

Shigella. Thirty-seven strains of *Shigella* were tested for sensitivity to both the parent strain and the purified *P. pestis* phage. Included were 26 strains of

S. paradysenteriae, 4 of *S. sonnei*, 3 of *S. dysenteriae*, 2 of *S. ambigua*, 1 of *S. alkalescens*, and 1 of *Shigella* sp., Newcastle type. Clear-cut lysis occurred with 4 strains, namely, *S. paradysenteriae* type 103, no. 12, *S. sonnei* no. 7, *S. dysenteriae* no. 44, and *S. ambigua* no. 33. Partial lysis occurred with *S. sonnei* cultures no. 5 and no. 6. All these 6 cultures showed secondary growth after 8 to 24 hours at 37 C. There was little difference between sensitivity to the parent phage and to the purified strain.

The 6 *Shigella* cultures which showed lysis were old stock cultures with the exception of *S. paradysenteriae* no. 12, which had been isolated from a dysentery case within a year. The *S. sonnei* cultures were all in the rough phase. Those strains of *Shigella* which were not sensitive to the phage included both recently isolated and old stock strains in both the rough and smooth phases. Five cultures of *S. paradysenteriae*, type 103, were not susceptible to the phage.

TABLE 2

Lysis of P. pestis and Shigella by parent phage and adapted phages

(Highest dilution showing confluent lysis on plates)

ORGANISM TESTED	PHAGE				
	Parent strain	Parent strain after 6 to 8 transfers on			
		S. paradysenteriae	*S. sonnei*	*S. shigae*	*S. ambigua*
P. pestis A1122	10^{-6}	10^{-6}	10^{-5}	10^{-5}	10^{-6}
S. paradysenteriae	10^{0}	10^{-3}	10^{-1}	10^{-2}	10^{-1}
S. sonnei	10^{0}	10^{0}	10^{-2}	10^{-1}	10^{0}
S. shigae	10^{-1}	10^{-3}	10^{-2}	10^{-3}	10^{-3}
S. ambigua	10^{-3}	10^{-3}	10^{-2}	10^{-3}	10^{-5}

Hence the parent phage failed to act on all members of a given species or even of a given serologic type.

Transfer of phage in broth cultures of Shigella. The parent strain of *P. pestis* phage was transferred serially on young broth cultures of each of the 4 *Shigella* cultures showing clear-cut lysis on plates. In the first two or three transfers the lysis in broth was slight and secondary growth developed within 4 hours. Later transfers gave complete lysis within 2 hours, and secondary growth was either absent or delayed for at least 18 hours. The phage was transferred in this manner for 25 subcultures on *S. paradysenteriae* no. 6, *S. ambigua* no. 33, and *S. sonnei* no. 7. Several attempts to transfer the phage beyond the ninth subculture on *S. shigae* no. 44 were unsuccessful. The purified strain of *P. pestis* phage was similarly transferred on *S. ambigua* no. 33.

The titer of the phage after transfer on *Shigella* was tested by the plate method and compared with the titer of the parent strain. The results are summarized in table 2.

Table 2 shows a 100-fold to a 1,000-fold increase in the activity of the phage after transfer on a particular *Shigella* species. The activity of the adapted phage for other sensitive *Shigella* strains sometimes increased, but not to the same degree as for the species on which the transfers were made. There was no increase in titer for *S. ambigua* except when the phage was transferred on that culture. No significant change in the titer for *P. pestis* occurred, which agrees with the results following adaptation of the parent phage to *P. pseudotuberculosis*. Results similar to these were obtained with the purified phage.

The phage apparently became adapted to the *Shigella* cultures so that it lysed them more readily than did the parent strain, but without any alteration of its ability to lyse *P. pestis*. This increased action on *Shigella* reached a maximum after four or five transfers, and subsequent subcultures up to 25 did not enhance its ability to lyse *Shigella* strains. The same observations were made regarding the phage adapted to *P. pseudotuberculosis*, which reached its maximum ability to lyse that organism in a few transfers and did not increase after additional passages.

The 41 cultures of *Shigella* which were not lysed by the parent strain of phage were tested again in contact with phage adapted to susceptible *Shigella* strains. Two additional strains of *S. paradysenteriae* and one of *S. sonnei* were lysed, but other *Shigella* cultures remained resistant.

Three *Salmonella* strains which were susceptible to the parent *P. pestis* phage were tested for sensitivity to the phage adapted to *Shigella*. Sensitivity was the same as for the parent phage.

Fourteen strains of *P. pseudotuberculosis* were tested against the phage adapted to *Shigella*. Sensitivity was essentially the same as for the parent strain of *P. pestis* phage. Adaptation of the parent phage to *P. pseudotuberculosis* did not alter its ability to lyse susceptible *Shigella*.

After transfer on *Shigella*, the adapted phage was again subcultured for six passages on *P. pestis* A1122. There was no change in its activity against either *P. pestis* or susceptible *Shigella* strains.

Transfer of phage on plate cultures of Shigella. Isolated plaques from plate cultures of the parent strain of phage on the different susceptible *Shigella* cultures were transferred for several generations, using the method of picking isolated plaques to broth described above. These "purified" phages were then tested against *P. pestis*. Size and number of the plaques were unchanged, and it was apparent that cultivation of the phage on *Shigella* did not alter its action on *P. pestis* in any observable way.

Both the parent strain and the purified strain of *P. pestis* phage produced plaques varying in size from 0.5 to 4.0 mm on susceptible *Shigella* cultures. The smaller plaques tended to increase in size on further incubation, but not so rapidly as did plaques on *P. pestis*. Small plaques (0.5 to 1.0 mm) and large plaques (3.0 mm or more) were isolated and tested for several generations. On plates with susceptible *Shigella* cultures, neither the large nor small plaques bred true, forming both large and small plaques. On plates of *P. pestis*, both large and small plaques produced uniformly large plaques identical with those produced by the parent strain of phage.

The *S. ambigua* culture was lysogenic for 20 strains of *Shigella*, but not for *Pasteurella*. This phage carried by *S. ambigua* was readily distinguished from *P. pestis* phage because of the minute plaques (0.1 to 0.3 mm) produced on *Shigella*.

Miscellaneous bacteria. The parent strain of *P. pestis* phage was tested by the plate method against 77 cultures, which included 37 species. The following 17 genera were represented: *Aerobacter*, *Alcaligenes*, *Bacillus*, *Chromobacterium*, *Corynebacterium*, *Eberthella*, *Escherichia*, *Gaffkya*, *Klebsiella*, *Micrococcus*, *Neisseria*, *Proteus*, *Pseudomonas*, *Serratia*, *Staphylococcus*, *Streptococcus*, and *Vibrio*. There was no lysis of any of these cultures. Since only one to six strains of each species were tested it cannot be assumed that other strains might not be susceptible to lysis.

SEROLOGICAL EXPERIMENTS

Burnet (1927) found a close correlation between the sensitivity of different bacteria to a phage and the distribution of surface somatic antigens. Numerous observations have supported the belief that sensitivity to phage is closely related to antigenic structure. The subject is well summarized in Topley and Wilson (1946).

In this study an attempt has been made to correlate the sensitivity of certain *Salmonella* and *Shigella* strains to *P. pestis* phage with a demonstrable antigenic relationship as shown by agglutination tests. It was considered unnecessary to investigate *Pasteurella* strains extensively, since the relationship in this genus has been thoroughly studied, especially by Schütze (1928, 1932) and by Bhatnagar (1940).

Methods. Standard macroscopic agglutination tests were made, using 0.3 per cent salt solution as the diluent. All tests were incubated overnight at 37 C.

Results

P. pseudotuberculosis. An antiserum prepared in rabbits by repeated injections of living *P. pestis* A1122 agglutinated both *P. pestis* and *P. pseudotuberculosis* (Saranac and Spokane strains) to about the same titer. These two strains of *P. pseudotuberculosis* were also agglutinated in dilutions up to 1:320 by antisera for *S. dysenteriae* and for *S. schottmuelleri*.

Salmonella. Considerable difficulty was experienced in serological studies owing to the roughness of the three *Salmonella* strains sensitive to *P. pestis* phage. However, there was definite indication of agglutination of *S. schottmuelleri* (no. 6) and *S. hirschfeldii* (58G, no. 31) in low titer by serum prepared against living, intact plague organisms, with satisfactory controls. No agglutination was observed using an antiserum for the carbohydrate fraction of *P. pestis*.

An antiserum prepared against *S. schottmuelleri* agglutinated *P. pestis* A1122 antigens in significant titer. The highest titers, 1:640, were obtained using antigens without envelope substance. Antigens prepared under conditions permitting the maximum development of envelope were not agglutinated by this

Salmonella antiserum. The antigenic factor in common may be considered, therefore, to be present in the soma of *P. pestis*, a reasonable hypothesis, since phage action is probably dependent on the surface antigens of the bacterial cell.

It seemed of value to determine whether the antigen or antigens held in common by *P. pestis* and *Salmonella* organisms could be identified. Efforts were unsuccessful, using alcoholized, heated, and live antigens of *P. pestis* A1122 with purified *Salmonella* "O" and "H" typing antisera. However, confirmation of agglutination of *P. pestis* by unpurified *Salmonella* typing antisera was supplied by the Division of Laboratories of the California State Department of Public Health. It seems likely that the common antigen is some minor somatic fraction as yet unrecognized.

Shigella. Antisera for *S. dysenteriae*, *S. ambigua*, and the W, X, Y, and Z types of *S. paradysenteriae* agglutinated *P. pestis* A1122 in dilutions of 1:40 to 1:320. Antiserum for the V type of *S. paradysenteriae* did not react with *P. pestis*. All of these antisera agglutinated the homologous *Shigella* strain in a titer of at least 1:2,560.

Antiserum for living, intact *P. pestis* agglutinated the majority of the 37 *Shigella* antigens in dilutions of 1:20 to 1:80, and 12 of the antigens in dilutions of 1:160 to 1:320. The results were identical with living and formalin-killed antigens. Antiserum for the carbohydrate substance of *P. pestis* agglutinated most of the *Shigella* cultures in 1:20 to 1:40 dilutions and three of the strains in 1:160 dilution. The titer of both these antisera for *P. pestis* was 1:1,280. The slight cross reactions in agglutination tests between *P. pestis* and *Shigella* suggest that they possess some common antigenic factor, although the majority of the *Shigella* strains tested were not acted upon by *P. pestis* phage. There was no correlation between the agglutination titer with *P. pestis* antiserum and lysis by *P. pestis* phage. For example, strains of *Shigella* agglutinated in 1:320 dilution might not be lysed by *P. pestis* phage, whereas others agglutinated only in 1:20 dilution might show lysis. This may be interpreted as a lack of correlation between phage sensitivity and agglutination, or as the result of an antigenic variation causing temporary loss of the minor relationship apparently existing between the two genera.

MISCELLANEOUS OBSERVATIONS

It was determined that lysates of *P. pestis* cultures were toxic for mice, producing death a few hours after intravenous inoculation. Similar observations were made with lysates of *P. pseudotuberculosis*, which were shown to be lethal for mice, guinea pigs, and rabbits. However, some strains of *P. pseudotuberculosis* showed a markedly greater toxigenic ability than others. Investigations are now being conducted to determine the properties of the toxin present in lysates of this organism and the factors responsible for the variation in toxigenic ability of different cultures.

Lysates of *P. pestis* were shown to be efficient vaccines, protecting mice against lethal doses of virulent plague bacilli. Flu (1929) made similar observations regarding the ability of such lysates to protect rats against many times the normal lethal dose of plague organisms. The use of lysates of *P. pestis* offers a number of interesting possibilities for human vaccination.

DISCUSSION

Flu (1927) observed that a phage isolated from canal water in Leyden, Holland, was able to lyse *P. pestis*, *E. coli*, and *S. dysenteriae*. Girard (1943) confirmed Flu's results, using several *P. pestis* phages. No other observations of this type have been recorded, although Girard (1942) has reported lysis of some strains of *P. pseudotuberculosis* by *P. pestis* phage.

If the assumption is made that the antigenic structure of the bacterial cell is one of the main factors permitting phage action (Topley and Wilson, 1946), the findings reported here apparently demonstrate a minor relationship between *P. pestis* and some strains of certain species of the *Salmonella* and *Shigella* groups. However, all strains of a given species, and even all strains of a given serological type within a species, were not equally susceptible to *P. pestis* phage. The organisms susceptible to *P. pestis* phage were not shown to be any more closely related to *P. pestis* than phage-resistant organisms, by the use of direct agglutination tests. Schütze (1928) has shown a close similarity between one of the somatic antigens of *P. pseudotuberculosis* and the "O" antigen of *S. schottmuelleri* and related salmonellas. No such relationship seems to have been described for *P. pestis*.

In view of known antigenic factors common to *P. pseudotuberculosis* and *P. pestis* (summarized by Schütze, 1932, and Bhatnagar, 1940), the action of *P. pestis* phage on *P. pseudotuberculosis* is not unexpected. The fact that strains of *P. pseudotuberculosis* differ so markedly in susceptibility to *P. pestis* phage (table 1) indicates an antigenic flexibility that may account for the wide variation in results obtained by different workers who have attempted to immunize animals against plague by the use of *P. pseudotuberculosis* antigens (summarized in Topley and Wilson, 1946). The action of *P. pestis* phage on certain *Shigella* and *Salmonella* organisms may possibly be accounted for by the presence of some unstable minor antigenic fraction held in common. The agglutination tests reported in this study give some basis for assuming the existence of such a relationship, but adequate proof is lacking.

ACKNOWLEDGMENTS

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SUMMARY

A strain of *Pasteurella pestis* phage which lysed 12 out of 12 strains of *P. pestis* has been shown to lyse, in varying degree, 19 out of 27 strains of *Pasteurella*

pseudotuberculosis. After adaptation to *P. pseudotuberculosis*, the phage lysed all 27 strains of that organism, most of them in higher dilution than originally. Other *Pasteurella* species were not susceptible to either phage.

Three out of 42 *Salmonella* strains and six out of 37 *Shigella* cultures were susceptible to *P. pestis* phage. Seventy-seven cultures from 17 other genera were not affected by the same phage. After adaptation to *Shigella* species, the phage showed an increased potency toward the six susceptible *Shigella* strains.

After adaptation to *P. pseudotuberculosis* or to *Shigella*, the phage retained completely its ability to lyse *P. pestis*.

Minor serological relationships have been shown by agglutination tests to exist between *P. pestis* and certain strains of *Salmonella* and *Shigella*. These relationships were not clearly correlated with susceptibility to phage.

The significance of these findings is discussed. The use of *P. pestis* lysates as vaccines and the study of the lethal properties of *P. pseudotuberculosis* lysates are suggested as worthy of further investigation.

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LIZARDS AS CARRIERS OF SALMONELLA AND PARACOLON BACTERIA

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In a previous paper the writers (1946) reported the isolation of *Salmonella montevideo* from a gila monster, and *Salmonella manhattan* from an iguana. In this paper we wish to record the isolation of *Salmonella rubislaw* and two sucrose-fermenting paracolons that possess *Salmonella* antigenic components from apparently healthy Pacific fence lizards (*Sceloporus occidentalis-occidentalis*).

Salmonella rubislaw. A total of 12 of these small lizards were caught within a radius of a quarter of a mile on the University campus at Davis. All were killed for autopsy, dissected aseptically, and their tissues seeded into tetrathionate broth (Difco plus 1:50,000 brilliant green). Subsequent bacteriological examination resulted in the isolation of *S. rubislaw* from three of them. One of these three lizards was found in the stomach of a gopher snake, and the other two were caught together 4 months later about a quarter of a mile away. The snake from which the first lizard came did not yield *S. rubislaw*, but this type was previously isolated from a gopher snake caught in the same vicinity (Hinshaw and McNeil, 1945). From two of the lizards, the organism was isolated from pooled viscera containing liver, spleen, lungs, and intestines. The third culture was isolated only from the intestine. Pooled liver, heart, and spleen cultured separately did not yield the organism.

Paracolon A. From one of the lizards which yielded *S. rubislaw*, an anaerogenic, gram-negative, motile bacterium, which on original isolation fermented glucose, sucrose, maltose, and dulcitol, but not lactose, was isolated. On antigenic analysis, the O antigens of this culture were shown to be identical to *Salmonella newport* VI, VIII. This conclusion was made after reciprocal agglutinin absorption tests with *S. newport* and the paracolon A antiserums. There was no evidence that the cultures contained the I, XIV, XXIV, or XXV components of *Salmonella carrau* or *Salmonella onderstepoort*, as did similar cultures from man described by Saphra and Silberberg (1942) and Saphra and Wassermann (1945).

Flagellar antigens were agglutinated by *Salmonella bonariensis* phase 1 antiserum (i) with a partial reaction only, but with a complete reaction with *Salmonella typhi* (d) antiserum. After passage through a week-old chick, complete agglutination was obtained with both the d and i antiserums. After suppression with the d antiserum, the H antigen of the original culture reacted with the i antiserum, but it has been difficult to maintain the i factor in cultures.

Reciprocal agglutinin absorption tests were made with *S. typhi* (d), *S. bonariensis* phase 1 (i), and paracolon A (culture 1181) antiserums and H type antigens. From the results recorded in table 1 it would appear that d and i are the major

H type antigenic components in this culture. All attempts to secure an additional phase by the antiserum suppression technique have failed to reveal additional antigenic components.

Detailed cultural studies of two strains (1181, 1182) yielded the following results: they were motile, gram-negative rods that were methyl-red-positive and Voges-Proskauer-negative. Urease medium (BBL) was not attacked. On T.S.I. (BBL) medium both the butt and slant gave an acid reaction, no gas production was noted, and there was no blackening of the medium. Though there was no evidence of H_2S production in T.S.I. medium, abundant production was noted in proteose-peptone ferric citrate agar and in cysteine gelatin. Gelatin was not liquefied in the period of observation (72 hours at 37 C and 2 months at room temperature). Both strains were indole-negative; gave an acid reaction in Jordan and Harmon's *d*-tartrate agar; and grew poorly in Koser's citrate broth. In potassium nitrate broth, nitrate was reduced to nitrite after 72 hours' incubation. Litmus milk became slightly acid in 24 hours, remained acid for 2 weeks, and then the litmus in the upper portion of the tubes became decolorized, but no curd developed. Growth in tetrathionate broth (Difco plus 1:50,000 brilliant green) was good. Growth on BBL desoxycholate agar, and on BBL desoxycholate citrate lactose sucrose (D.C.L.S.) agar was as good as on nutrient agar, but there was a tendency for both cultures to develop rough types. This tendency was also noted in all liquid media. Smooth cultures, satisfactory for antigens, were obtained by aid of the starch agar technique of Crossley, Ferguson, and Brydson (1946).

TABLE 1

Results of agglutinin absorption tests with H antiserums

FLAGELLAR ANTIGENS	ANTISERUMS						
	Paracolon A (1181)					*S. typhi* (d) + *S. bonariensis* (i)*	
		Absorbed by					Absorbed by
	Unabsorbed	*S. typhi* (d)	*S. bonariensis* (i)	Both *S. t.* and *S. b.* (d + i)	Paracolon A	Unabsorbed	Paracolon A
Paracolon A (1181)	25,600	100(±)	12,800	<100	<100	3200	<50
S. typhi (d)	25,600	<100	12,800	<100	100(±)	3200	<100
S. bonariensis (i)	400	400	<50	<50	<50	6400	<100

Figures indicate the highest dilution at which agglutination occurred.

* Equal parts of these antiserums were mixed for this test.

It was not possible to demonstrate gas production in any medium used, even though tubes were corked and kept under observation for over a month. Sucrose was rapdily fermented, as were the following: maltose, trehalose, glucose, galactose, arabinose, xylose, rhamnose, mannitol, dulcitol, and sorbitol. No reaction occurred, in the 30 days of observation, in lactose, inulin, salicin, and inositol. Glycerol became slightly acid in 48 hours, then became neutral for 3 weeks, and finally again showed a slight acid reaction (bromthymol blue).

Paracolon B. From the viscera of two of the lizards an aerogenic, sucrose-positive type was isolated. Its biochemical reactions were identical with the type described above except that it was aerogenic and liquefied gelatin. On antigenic analysis, however, the O antigens of both cultures (Pc219 and 220) were completely agglutinated by spot tests with *Salmonella typhimurium* and *Salmonella schleissheim* O antiserums, and partially with *Salmonella gallinarum* antiserum. No reactions were obtained with any of the other O type *Salmonella* antiserums, nor with any of our available paracolon antiserums. They did not react to single factor antiserums V or XXVII. This would indicate that the known O antigens are IV, XII. No H type antigenic components have been demonstrated by us.

DISCUSSION

The isolations reported in this paper were made in connection with a survey being conducted to determine all possible sources of *Salmonella* and paracolon infections in turkeys. Previous papers by the writers (1945; 1946) have incriminated snakes as possible transmitters. Lizards on exhibit at one zoo (McNeil and Hinshaw, 1946) suggested that some of the 51 known species of wild lizards in California might also be sources of infection. The results reported indicate that this may prove a fertile field for a *Salmonella* survey.

As far as our records show, the two sucrose-positive paracolons described herein have not been isolated from turkeys. We have, however, isolated other types from outbreaks of enteritis in turkey poults and young chicks and from adult survivors of these outbreaks. It is of interest that two of four 1-week-old White Leghorn chicks died after inoculation with paracolon A described above. Four such strains, which were recently isolated from adult turkey carriers from three different ranches in the same community, all had O antigens of *S. onderstepoort* and resemble similar ones described by Saphra and Silberberg (1942).

No attempt is made to classify these cultures, nor has any attempt been made to make a detailed study of the antigens of the second paracolon. From the information available the formula indicated for the anaerogenic type (A) is VI, VIII,:d, i . . . . The incomplete formula for the aerogenic type (b) is IV, XII–: . . . .

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SUMMARY

The isolation of *Salmonella rubislaw* (XI:r, enx) is reported from 3 of 12 Pacific fence lizards (*Sceloporus occidentalis-occidentalis*) examined.

From the same group of lizards two sucrose-positive paracolons which have

Salmonella antigenic components were also isolated. One of these, an anaerogenic type, has the *Salmonella* type formula VI, VIII,:d, i.... The other contains the O factors IV and XII of group B, but no H factors were identified.

Detailed biochemical studies of the paracolon types are given.

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THE PREPARATION OF GLYCOLYTICALLY ACTIVE WASHED CELLS OF LACTOBACILLI

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Lactobacillus casei is a useful organism for microbiologists and biochemists because it can be used in certain microbiological assays. Extensive investigations have been conducted and considerable information has been obtained concerning the nutritional requirements of this homolactic organism by measuring its growth response to various substances. On the other hand, investigations with washed cells of this organism have been surprisingly few in spite of the fact that experimental conditions with such cell preparations can be more readily defined and controlled than in studies conducted with growing organisms in culture media.

That the optimum growth conditions and nutritive requirements for the production of cells of a particular organism vary with the enzyme system to be studied has been well established. Thus, Gale (1940) found that cells of *Streptococcus faecalis* harvested from a trypsin-digested casein medium with a terminal culture pH below 5.0 possessed an active tyrosine decarboxylase system. Wood and Gunsalus (1942) found that cells of *Streptococcus mastitidis* grown in a tryptone, yeast extract, low glucose medium harvested before the culture pH had dropped below 6.8 displayed excellent glucose dehydrogenase properties. Bellamy and Gunsalus (1944) subsequently confirmed and extended Gale's findings, describing a medium, synthetic except for the incorporation of acid-hydrolyzed gelatin and a folic acid concentrate, which supported cells demonstrating good decarboxylation properties. Wood and his associates (1945) used *Lactobacillus casei* cells grown in a glucose, yeast extract medium to degrade glycogen hydrolyzates to lactic acid. Reithel (1946) prepared a cell-free extract of *Streptococcus thermophilus*, which fermented lactose and glucose to lactic acid in the presence of adenosine triphosphate (ATP), from cells grown on a medium containing Difco broth, lactose, and peptonized milk. The present study is an attempt to devise a medium which could be used to prepare fairly large amounts of washed cells of lactobacilli (which could be lyophilized or used in the preparation of cell-free extracts) which would actively ferment glucose to lactic acid. No attempt was made to obtain an intimate knowledge of the chemical composition of such a medium.

EXPERIMENTAL PROCEDURES

Organisms. *Lactobacillus casei* 7469, which is used in our laboratories for routine riboflavin assays, and *Lactobacillus arabinosus* 17-5, for nicotinic acid assays, were used in these studies.

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[1] With the technical assistance of Arlene M. Larson.

Preparation of cell suspensions. Media of varying composition were generally made up in 600-ml quantities in 1-liter Erlenmeyer flasks; 1.0 per cent inocula were used and the cultures were incubated for 16 to 20 hours at 37 C. After the cells were harvested by centrifugation, they were washed twice with about one-third the culture volume of chilled 0.5 per cent salt solution. The composition of the stock salt solution (used in our riboflavin assays), from which the chilled 0.5 per cent salt solution was prepared, was

$MgSO_4 \cdot 7H_2O$	4.0 per cent
NaCl	0.2 per cent
$FeSO_4 \cdot 7H_2O$	0.2 per cent
$MnSO_4 \cdot H_2O$	0.2 per cent

After the second washing, the cells were transferred to graduated centrifuge tubes and compacted by centrifugation. The volumes of the packed cells were read and chilled salt solution was added to make 1.0 per cent (by volume) cell suspensions. Kjeldahl N values were determined on aliquots of the 1.0 per cent suspensions.

Tests for glycolytic activity. The activities of cell suspensions from various media were measured in a Warburg apparatus by the usual methods. The flask concentrations and other pertinent experimental conditions were as follows:

Glucose	0.01 M
$NaHCO_3$	0.02 M
Adenosine triphosphate (ATP)	0.01 M
Salt solution	1.0 per cent of the stock salt solution
Cells	0.15 per cent by volume
Total volume	3.3 ml
Atmosphere	CO_2
pH	ca. 6.2
Temperature	ca. 37 C ± 0.01 C

Because Gunsalus and Niven (1942) have shown that at pH values above 6.5 homolactic streptococci produce formic acid, acetic acid, and ethanol instead of lactic acid from glucose, the pH of the reaction mixture was kept below this point. The gas evolution was followed for 180 minutes. Under these experimental conditions, the lactic acid produced is measured as carbon dioxide liberated. Preliminary experiments showed that the addition of both ATP and the salt mixture was necessary for the maximum production of lactic acid. From the Kjeldahl N values obtained on aliquots of the 1.0 per cent cell suspensions and the amount of carbon dioxide evolved in the Warburg apparatus, the amount of gas evolved per 0.1 mg N (cells) in 180 minutes was calculated. In this manner the effect of the variations between the densities of the various cell suspensions was obviated.

RESULTS

The medium of Wood and Gunsalus for the "production of active cells of streptococci" was selected as a guide because the streptococci and the lactobacilli are metabolically related, both being homolactic organisms. Their medium, made up with Difco tryptone, yeast extract, glucose, and K_2HPO_4, was first used in an attempt to grow glycolytically active *L. casei* cells. The medium supported heavy growths of organisms which displayed excellent dehydrogenase properties. Table 1 shows some of their dehydrogenase properties.

The tube concentrations were: cells (1:300 by volume); methylene blue (2.5×10^{-5} M); substrate (0.01 M); pH 7.3, K_2HPO_4-NaOH buffer (0.025 M); 0.5 per cent salt solution; and the total volume was 10 ml. The tests were run at 37 C with a stream of nitrogen (purified of oxygen by running it over heated copper) constantly bubbling through the reaction mixture to ensure anaerobic conditions and continued agitation so the cells would not settle out. Under these conditions, the cells were capable of using ethanol, glucose, pyruvic acid, lactic acid, and glycerol, but not formic and succinic acids, as hydrogen donors and methylene blue as the hydrogen acceptors.

TABLE 1

Dehydrogenase properties of cells

SUBSTRATE	METHYLENE BLUE REDUCTION TIME IN MINUTES
Ethanol	9
Glucose	39
Sodium pyruvate	43
Sodium lactate	74
Glycerol	90
Sodium succinate	210
Sodium formate	235
Water (control)	200

When these cells were tested in the Warburg apparatus, however, they were only slightly active in the breakdown of glucose to lactic acid. Therefore this medium was modified in attempts to devise one which could be used to grow glycolytically active cells.

Potassium dibasic phosphate. The effect of K_2HPO_4 in concentrations of 0.25, and 0.5 per cent was determined in a basal medium containing tryptone (1.0 per cent), yeast extract (1.0 per cent), and glucose (0.1 per cent). The initial pH of the cultures was adjusted to approximately 7.0 as measured by the glass electrode. The results are given in figure 1. As can be seen, the glycolytic properties of the cells were considerably increased by the removal of K_2HPO_4 from the medium. In subsequent experiments, K_2HPO_4 was not added to the medium.

In order to localize this peculiar effect of K_2HPO_4 to either the potassium or phosphate ion, *L. casei* was grown in a basal medium of tryptone (0.5 per cent), yeast extract (1.0 per cent), and glucose (0.5 per cent); the basal medium plus K_2HPO_4 (0.5 per cent); basal medium plus $Na_2HPO_4 \cdot 12H_2O$ (1.03 per cent), so that the phosphate concentration would be the same as the medium with the potassium salt; and basal medium plus KCl (0.43 per cent), so that the potassium concentration would be the same as in the medium with K_2HPO_4. The initial pH values of the media were adjusted to approximately 7.0. The results are given in figure 2.

As can be seen, both the K_2HPO_4 and Na_2HPO_4 adversely affected the glycolytic activity of the cells harvested from media to which they were added. These results were also obtained to an even more marked degree in experiments in which the basal medium was the same as that used for the K_2HPO_4 concentration effect. It should be noted and emphasized that neither K_2HPO_4 nor Na_2HPO_4 affect adversely the degree of growth of the cells in media to which they are added. As a matter of fact, the cell harvests from media with K_2HPO_4 were sometimes greater than those from the basal medium.

It was thought that possibly this effect might be due to the phosphate-buffering effect. Since the terminal pH values of the cultures containing K_2HPO_4 and Na_2HPO_4 were 4.3 as compared to 4.0 and 4.1 for the KCl and the basal medium, respectively, there existed the possibility that lower pH values favored the development of glycolytically active cells, or, conversely, the higher pH conditions adversely affected the production of the glycolytic enzymes. Therefore cells were grown and harvested from media containing tryptone (0.5 per cent), yeast extract (1.0 per cent), and glucose (0.1 per cent), initially adjusted to pH 5.0, 5.5, 6.0, 6.5, and 7.0. The terminal pH values of these cultures were 4.7, 4.6, 4.6, 4.7, and 4.8, respectively. The glucose concentration of 0.1 per cent was used instead of 0.5 per cent because, if lower pH values do favor the development of glycolytic enzymes, there was the possibility that the higher sugar concentration, and thus greater acid production, might cover up the effect of adjusting the initial pH of the cultures to the higher values by rapidly lowering the pH. However, the results shown in figure 3 show that such was not the case. The lower the initial pH of the cultures, the lower were the activities and also the cell harvests.

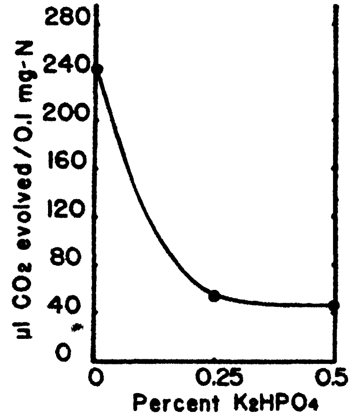

FIG. 1

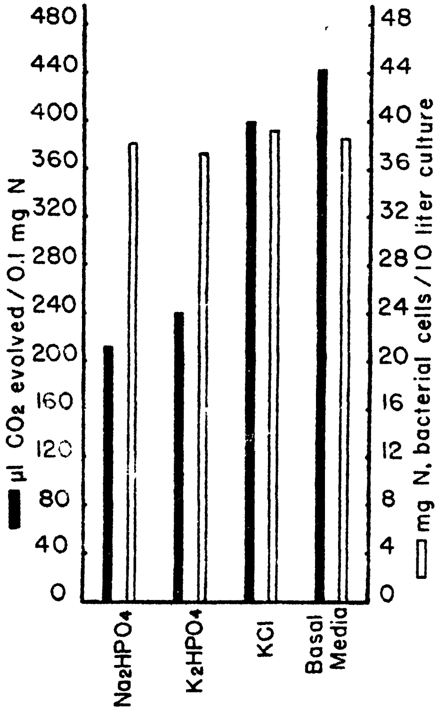

FIG. 2

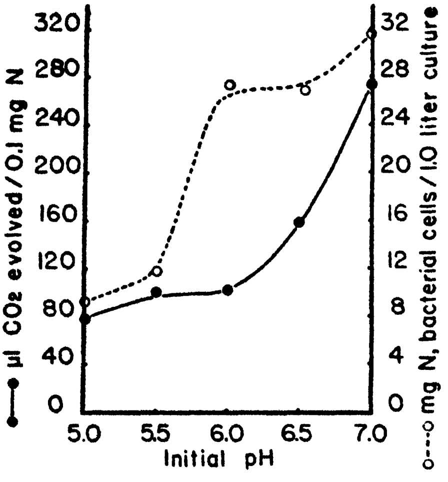

FIG. 3

At present we are unable to account for this phosphate effect. It will be noted that K_2HPO_4 is missing from the media which Wood and his associates used to produce *L. casei* cells to degrade glycogen hydrolyzates to lactic acid.

Glucose. The effect of glucose in 0, 0.1, 0.5, 1.0, and 2.0 per cent concentration on the activity of *L. casei* cells was determined in a basal medium of tryptone (1.0 per cent) and yeast extract (1.0 per cent). The terminal pH values were 5.4, 4.2, 4.0, and 4.0, respectively. The media were initially adjusted to pH 6.8 to 7.1. The results are given in figure 4. Apparently the presence of glucose or the breakdown products of this hexose in the medium is essential for the development of at least one of the enzymes involved in the glycolysis of glucose by

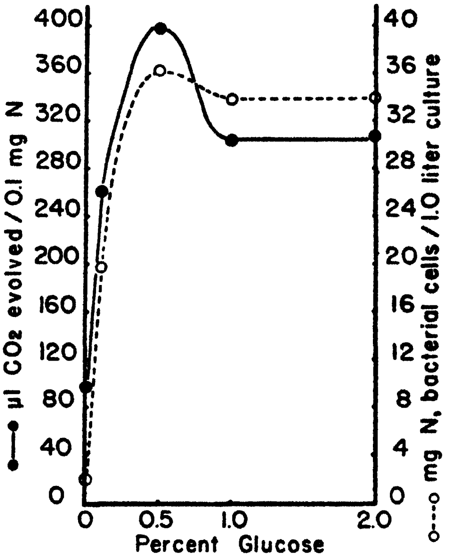

FIG. 4

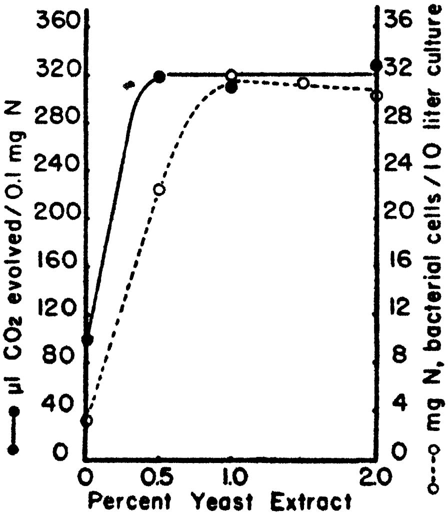

FIG. 5

this organism. This might be taken as evidence of the "adaptive" nature of certain of the mechanisms playing roles in glycolysis.

Yeast extract. The effect of 0, 0.5, 1.0, and 2.0 per cent yeast extract on the activity of *L. casei* cells was measured in a basal medium of glucose (0.5 per cent) and tryptone (1.0 per cent). The terminal pH values were 5.4, 4.2, 4.0, and 4.0, respectively. The media were initially adjusted to pH 6.8 to 7.1. The results are given in figure 5. Cells grown and harvested from 0.5 per cent yeast extract media were as active as those obtained from the 2.0 per cent media. However, the cell harvest was greater from media containing at least 1.0 per cent yeast extract so this was considered to be the optimum amount.

Tryptone. The effect on *L. casei* cells of 0, 0.5, 1.0, and 2.0 per cent tryptone was measured in basal medium containing glucose (0.5 per cent) and yeast extract (1.0 per cent). The terminal pH values of the culture were 3.9, 4.0, 4.1, and 4.1, respectively. Initially the media were adjusted to pH 6.8 to 6.9. The results are given in figure 6. As will be noted, the glycolytic activity and the cell harvest curves are practically mirror images of each other. The higher the concentration of tryptone, the greater is the cell harvest but weaker the glycolytic activity. Apparently the glycolytic enzymes are "diluted out" by the larger number of cells. In terms of the maximum amount of glycolytic enzymes per 1.0 liter of medium, 0.5 per cent concentration of tryptone seems to be the most satisfactory. However, medium containing 2.0 per cent tryptone might also be satisfactory depending upon the purposes for which the cells are grown.

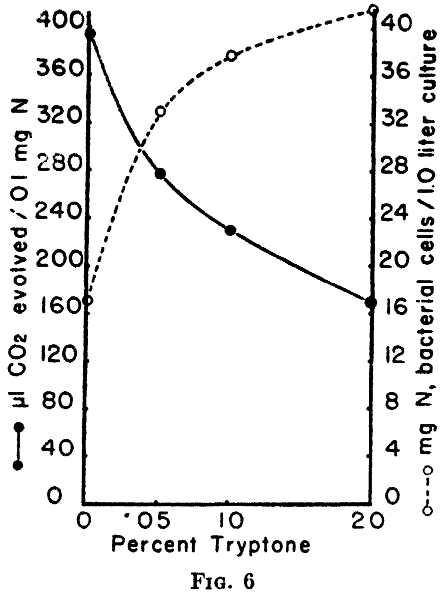

FIG. 6

Lactobacillus arabinosus. The strain of *Lactobacillus arabinosus* 17-5, used routinely in our laboratory for the microbiological assay of nicotinic acid, was also grown in medium containing glucose (0.5 per cent), yeast extract (1.0 per cent), and tryptone (0.5 per cent), with an initial pH of 7.0. Although the growth was not so luxuriant as that of *Lactobacillus casei*, fairly active cells were obtained. From 1.0 liter of culture the cell harvest was cells with a Kjeldahl N value of 10.7 mg (as compared to values of approximately 32 mg with *L. casei*). At the end of 3 hours, the gas evolution ranged from approximately 210 to 250 microliters of carbon dioxide.

Lyophilization. Cells of *L. casei* harvested from media containing glucose 0.5 per cent), yeast extract (1.0 per cent), and tryptone (0.5 and 2.0 per cent) and dried from the frozen state displayed fair glycolytic properties. The gas evolution per 1.0 mg N of bacterial cells (0.5 ml of a 1.0 per cent lyophilized cell suspension added to a Warburg flask containing a reaction mixture with a total volume of 3.3 ml) in 3 hours varied from 350 to 550 microliters depending on the conditions.

DISCUSSION

The Warburg method of determining the glycolytic activity of lactobacilli may not be so absolutely accurate as the direct estimation of lactic acid. It will be noted that Reithel found some discrepancies between the amount of lactic acid measured directly and indirectly by the manometric technique. However, the method does give a reliable measure of the relative glycolytic activities of homolactic organisms. The ease with which the activities of a number of cell preparations can be estimated is its principal recommendation.

The fact that cells which display excellent dehydrogenase properties do not necessarily possess comparably good glycolytic activity has been demonstrated. Wood and Gunsalus (1942) were not unaware of this possibility, for they were aware of the criticisms offered by Barron and Jacobs (1938), who warned of certain inherent weaknesses of depending solely on the use of the Thunberg method for measuring activity of cells. The evidence presented here does not contradict the work of Wood and Gunsalus. *L. casei* cells grown in their medium do display excellent dehydrogenase properties. The evidence does seem to indicate, however, that conditions under which cells with good dehydrogenase activity are produced are not the best, at least with some homolactic organisms, for the production of glycolytically active cells.

The effect of K_2HPO_4 was unexpected. As yet, we have no explanation for this peculiar phenomenon.

CONCLUSIONS

Under the experimental conditions described, the data seem to justify the conclusions which follow:

Washed cell suspensions of *Lactobacillus casei* and *Lactobacillus arabinosus* which are glycolytically active can be prepared from a medium containing glucose (0.5 per cent), yeast extract (1.0 per cent), and tryptone (0.5 to 2.0 per cent).

Despite the fact that the terminal pH values of the cultures drop as low as 3.9 or 4.0, the cells are still active.

K_2HPO_4 and Na_2HPO_4 at 0.5 per cent concentration do not inhibit the growth of the organisms in the culture but do affect adversely the activity of the cells harvested from the phosphate-containing media.

The cells harvested from the medium described and dried from the frozen state display fair glycolytic activity.

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ADAPTATION IN A YEAST UNABLE TO FERMENT GLUCOSE[1]

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In view of the high degree of specificity exhibited by strains of *Saccharomyces cerevisiae* with respect to the ability to metabolize different sugars and the occurrence of types with loss of the ability to grow on a specific sugar, it would seem that ascospores could be obtained occasionally the metabolic capacities of which would be limited to hexose degradation products and would not include the ability to utilize glucose. As far as we know, no such strain has been reported. This may be because these strains are ordinarily eliminated by the choice of nutrient media (see below). This paper reports on the existence of yeasts incapable of utilizing sugar (glucose) and provides some data on their behavior.

Isolation of non-glucose-utilizing yeasts. Spores from ten different cultures were suspended in distilled water, heated at 58 C for 4 minutes to kill vegetative cells, and then plated on agar media containing organic acids in concentrations of 1 per cent in substitution for glucose in the synthetic medium including vitamins described by Burkholder (1943), and with alkali added to make the initial pH of the media approximately 6. After 4 days' incubation at 30 C, individual colonies were transferred to tubes containing liquid media of the same composition. After a few days sufficient growth occurred in these tubes to make loop transfers to tubes containing the standard synthetic medium with 2 per cent glucose. These tubes were incubated under CO_2 at 30 C. From over 400 such transfers from individual colonies 12 were found which grew on the organic acid substrates, i.e., on lactate, succinate, or malate, or on combinations of salts of these acids, but which failed to grow on glucose. Subcultures were maintained on lactate medium and were tested repeatedly for their ability to grow on glucose either in the presence or absence of atmospheric oxygen. It was found that all but two of the twelve organisms quickly reverted to glucose utilization, and these were not further investigated. Nearly all the subcultures of one of the two remaining isolates reverted to sugar utilization within a week and generally within a few days after exposure to glucose in liquid medium. With rare exceptions, reisolations from the reverted cultures yielded only glucose-utilizing organisms.

The remaining culture, isolate no. 97, was obtained from an ascospore of a hybrid of the following constitution: *Saccharomyces microellipsoideus* (NRRL no. Y-1350) × *S. cerevisiae* (Mrak 93).[2] Culture no. 97 has been carried for 3 months on a lactate medium and has been kept stable with respect to nonutilization of glucose by frequent transfers to new medium. It is unable under the conditions of these experiments either to ferment or respire glucose (at least at a significant rate). The same holds for fructose and sucrose. As will be seen below, however, this stability would not be maintained in standard culture practice.

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[1] This work was supported by a grant from Anheuser-Busch, Inc., St. Louis, Missouri.

[2] The NRRL culture was obtained from Dr. L. J. Wickerham, Northern Regional Research Laboratory at Peoria, and the Mrak culture was obtained from Dr. E. M. Mrak of the University of California, Berkeley.

Adaptation to glucose utilization. In tests of the stability of failure to utilize glucose, loop transfers were made from the lactate medium to synthetic medium with glucose. Many such transfers of strain no. 97 after standing for some weeks would show a sudden rapid growth and fermentation; i.e., a reversion to glucose utilization occurred in individual tubes after long and varying periods of incubation in contact with the substrate.

On the other hand, it was observed that the addition of glucose to the organic acid substrates tended to repress the growth of the organism. Thus, not only is glucose not assimilated by the unadapted organism, but it actually inhibits the utilization of the organic acids.

To study these phenomena in more detail, strain no. 97 was cultured on a series of media containing lactate or glucose in different concentrations or consisting of the two substances combined in different proportions and added to the synthetic medium. The growth in 10-ml volumes in 6-by-¾-inch test tubes was measured photometrically at suitable intervals. Some typical results are shown in figures 1A to 5A. For the sake of comparison corresponding results with an adapted strain, isolate no. 322, are included as parts B of the figures.

If the growth on media with lactate alone added is first considered, it may be seen (figure 1) that the growth of strain no. 97 is greater the lower the concentration of lactate between 4 and 0.5 per cent, whereas with the glucose-adapted strain no. 322 the reverse is true. The results with no. 97 might appear to mean that lactate is not utilized but rather interferes with the utilization of some other component of the substrate. However, the fact that only traces of growth are obtained in control tubes without lactate excludes this interpretation and indicates rather that the concentration of lactate no doubt tends to decrease both the growth rate and final yields. In the case of strain no. 322, on the other hand, even 4 per cent, the highest concentration used, may not be sufficient for optimum growth.

In a comparison of the growth on lactate with that on glucose (figure 2A) and on mixtures of glucose and lactate (figures 3A, 4A, and 5A), the following striking differences are to be noted:

1. In the presence of glucose there is a very long period between inoculation and the appearance of measurable growth, followed suddenly by a period of very rapid growth.

The starting time of this rapid growth varies greatly from one culture to the next. Some data for individual cultures are presented in table 1. These figures, the points of intersection of the abscissa with the slopes of the growth curves in the regions of rapid linear growth, represent approximately the periods required for adaptation to glucose utilization. In most cases they correspond to the times of appearance of sufficient yields to be measurable in the Klett photometer. They do not, however, correspond to the times of initiation of growth. Traces of yeasts, too small to measure with the instrument, are generally observable earlier, usually within 3 to 4 days after inoculation on the mixtures of lactate and glucose. Occasionally, as is illustrated by some curves in figures 2A and 5A and checked by loop transfers to glucose medium, considerable yields are obtained before adaptation to glucose occurs. On the average, 150 to 170 hours were required on mixtures of lactate and glucose, and 200 or more hours on glucose alone. One culture adapted itself after only 40 hours, but several failed to grow appreciably until after 10 or more days.

2. The time required from the beginning of rapid growth to the attainment of maximum rate is difficult to estimate exactly but is certainly also very variable from one culture to the next. The figures for individual cultures compiled in table 2 give average values from 37 to 68 hours for the different sets to reach the flex points of the curves. Perhaps the length of time required increases with the concentration of lactate in the medium, but this tendency is certainly less marked than the extreme differences exhibited between individual cultures of a given set, which will be discussed below.

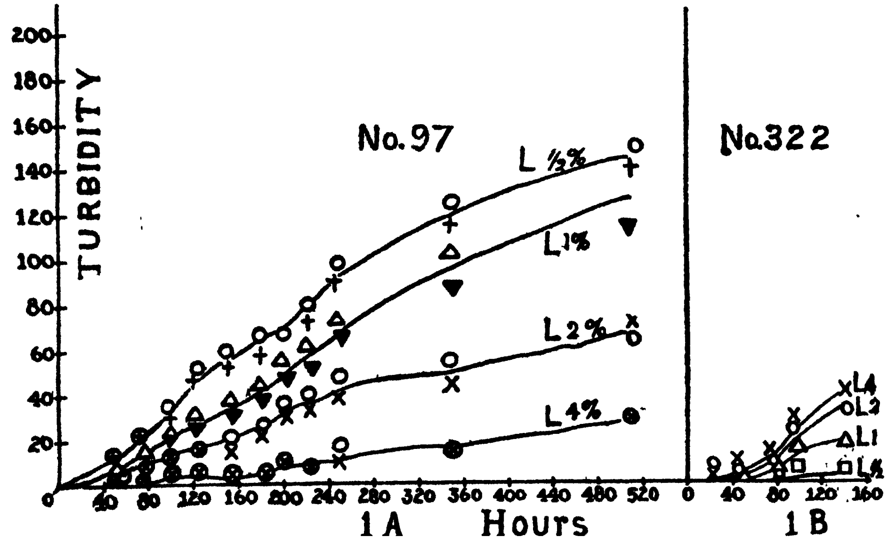

FIG. 1

A. Growth of eight subcultures of no. 97 (previously maintained on lactate) on different concentrations of lactate in synthetic medium. Ordinates, density of yeast suspensions; abscissae, hours.

B. Growth of four subcultures of no. 322 under similar conditions.

3. The later portions of the curves show that with the addition of glucose alone (figure 2) to the medium the growth approaches the final yields very quickly, once it has started, whereas with mixtures of lactate and glucose (figures 3 to 5) the growth decreases very markedly, as a rule, long before the final yields are attained. This decrease in growth rate evidently is due to a number of different causes, which operate to different extents in individual cultures. In the first place, where adaptation to glucose has occurred before the lactate has been de-

pleted, subsequent growth may result from further utilization of the remaining lactate. (See curves 1 and 3 in figure 3A; curves 1 and 2 in figure 4A; and curves 1, 2, 4, and 5 in figure 5A.) Secondly, it is clear from a comparison of the figures with increasing lactate concentrations that there is a correspondingly slower rate

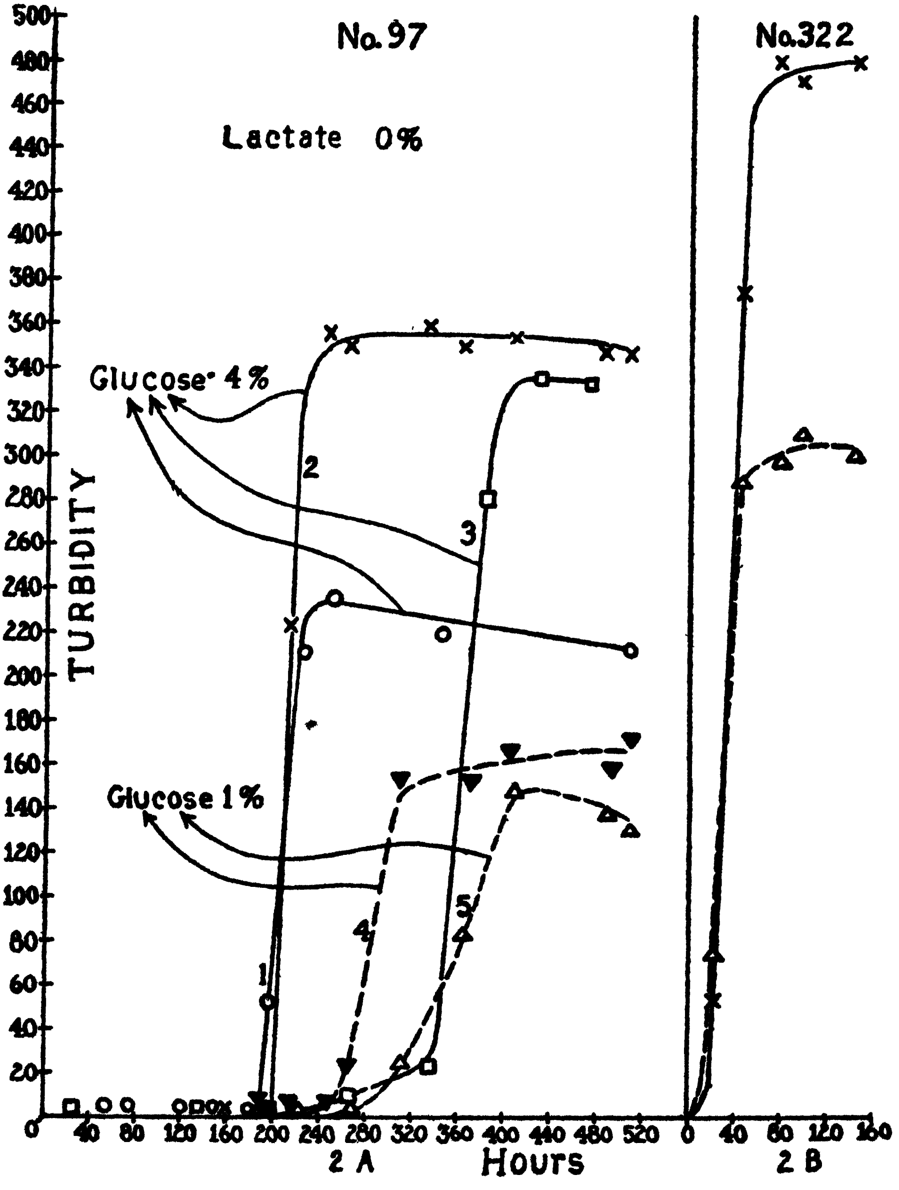

FIG. 2

A. Growth of subculture of glucose-unadapted strain no. 97 in synthetic medium containing no lactate. Solid lines, 4 per cent glucose; broken lines, 1 per cent glucose.

B. Growth of subcultures of the adapted strain no. 322 on glucose.

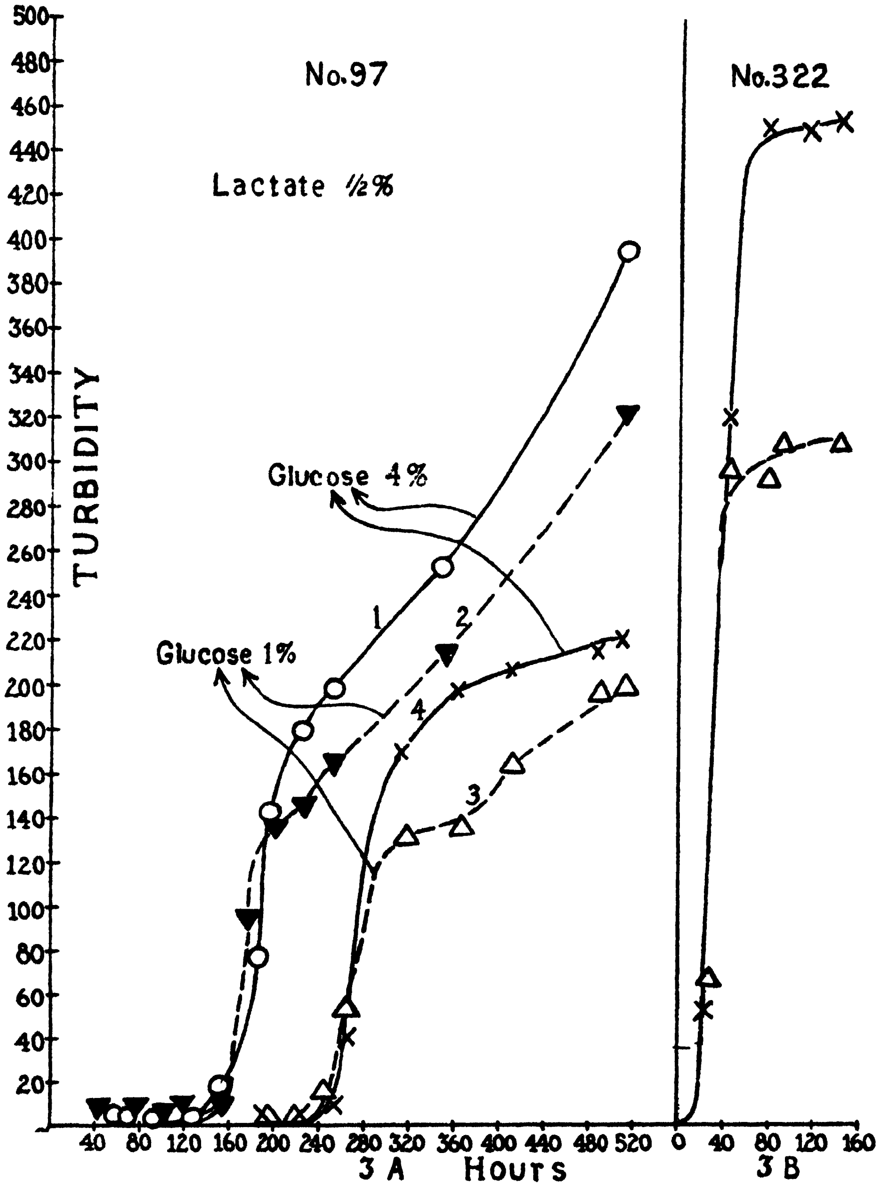

Fig. 3

A. Growth of subcultures of the glucose-unadapted strain no. 97 in a mixture of glucose and 0.5 per cent lactate.

B. Corresponding growth of the adapted strain no. 322. Solid lines, 4 per cent glucose; broken lines, 1 per cent glucose.

of utilization of the glucose and that the growth rates frequently begin to decrease long before the glucose has been depleted, i.e., inhibition occurs when the amount of growth is only a fraction of the final yield, so that glucose as well as the lactate

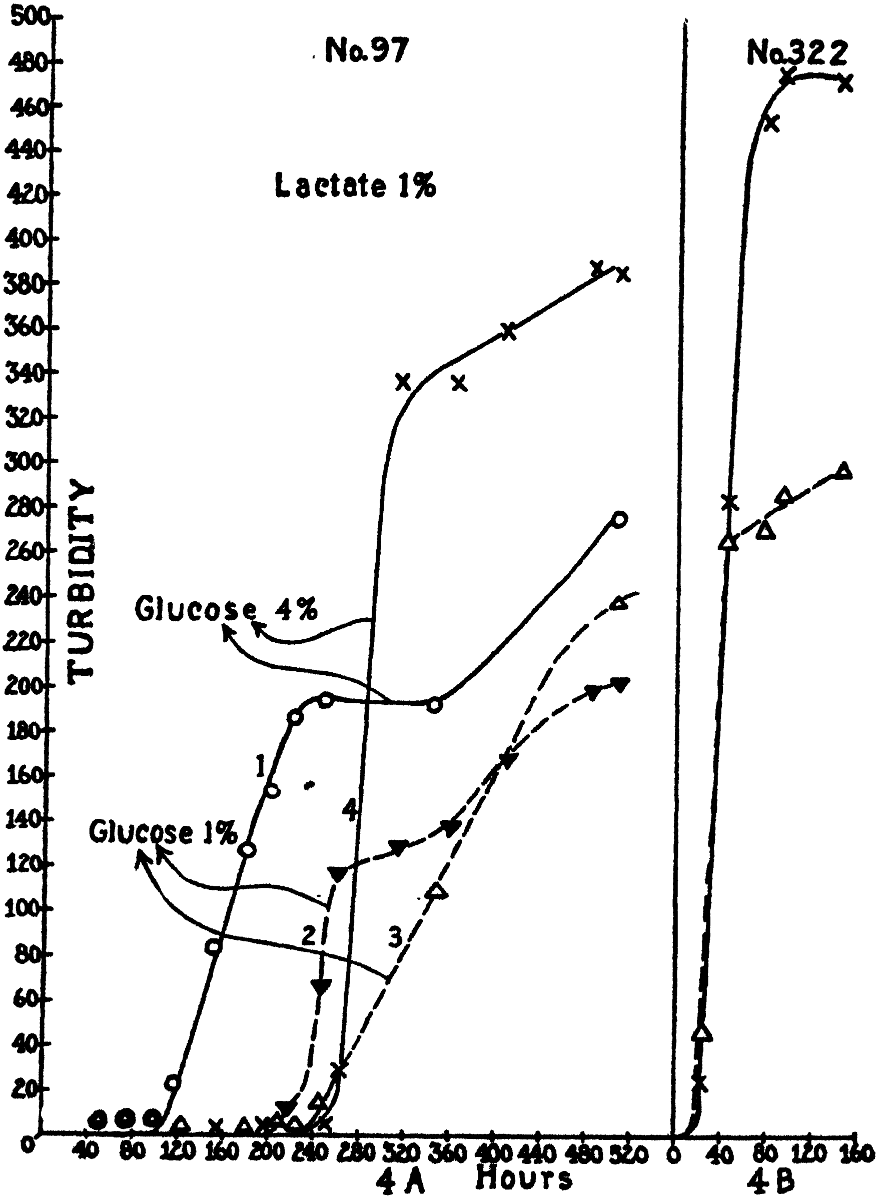

FIG. 4

A. Growth of subcultures of the glucose-unadapted strain no. 97 in a mixture of glucose and 1.0 per cent lactate.
B. See explanation, figure 3B.

present must be required for the subsequent yield. This decrease in growth rate before the final yield is attained, with increasing concentration of lactate, also holds for strain 322. It appears, therefore, that high lactate concentrations

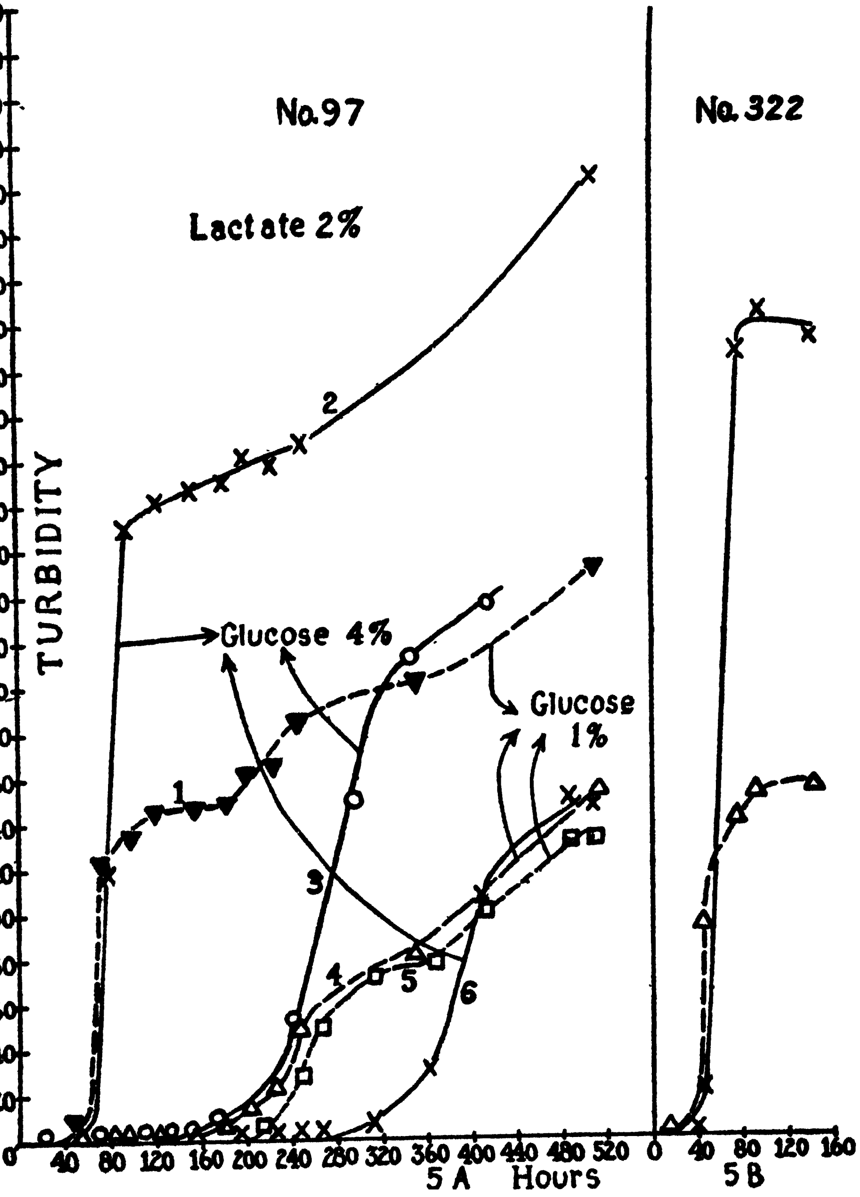

FIG. 5

A. Growth of subcultures of the glucose-unadapted strain no. 97 in a mixture of glucose and 2.0 per cent lactate.

B. See explanation, figure 3B.

interfere to some extent with the utilization of glucose. Hence, the phase of subsequent slow growth and the secondary rise in growth rate may be due to an adaptation to glucose utilization in the presence of lactate, i.e., to the develop-

TABLE 1

Adaptation times

CULTURE NO.	MEDIUM							
	G_4 L_2	G_4 L_1	G_4 L_2	G_1 L_2	G_1 L_1	G_1 $L_{\frac{1}{2}}$	G_4	G_1
97 1		240	250	230	210	240	420	300
97 2	290	260	240	210	210	210	200	250
97 3	55	130	150	40	190	130	330	230
97 4	250	110	110	170	230	190	440	190
97 5	130	130		70	130	150	390	120
97 6	120	120	(600)*	50	130	120	280	280
97 7	105	195	110	130	180	210	220	190
97 8	170	110	130	130	130	100	110	150
Mean	157	162	163	129	176	169	299	214
322	25	10	10	25	10	20	10	10

Time in hours from inoculation to initiation of rapid growth in individual cultures on mixtures of lactic acid and glucose and on glucose.

L = lactic acid; G = glucose; subscripts = concentrations in grams per 100 ml medium.

* Did not start rapid growth even though live cells were present.

TABLE 2

Periods of increasing growth rates

CULTURE NO.	MEDIUM							
	G_4 L_2	G_4 L_1	G_4 $L_{\frac{1}{2}}$	G_1 L_2	G_1 L_1	G_1 $L_{\frac{1}{2}}$	G_4	G_1
97 1		20	40	30	20	20	20	60
97 2	90	70	40	40	30	50	15	30
97 3	25	60	40	20	40	40	80	70
97 4	70	40	30	50	120	50	20	70
97 5	50	90		130	60	30	50	70
97 6	60	100	—*	130	25	25	70	90
97 7	70	55	30	130	60	50	150	100
97 8	80	40	50	90	40	30	50	60
Mean	63	59	(38)	66	48	37	57	69
322	25	20	20	15	25	30	20	20

Time in hours from initiation of rapid growth to attainment of maximum rate (flex point of curves) of individual cultures on mixtures of lactic acid and glucose.

L = lactic acid; G = glucose; subscripts = concentrations of substrate in grams per 100 ml of medium.

* Did not start rapid growth.

ment of a tolerance to lactate as well as to the utilization of lactate. Numerous other changes must occur in the medium that may impede the rate and also, in specific instances, give preference to the rapid increase of mutant types in the cultures.

4. The final total yield of a culture is primarily determined by the glucose content of the medium. Thus the largest yields on the average were obtained on 4 per cent glucose, and approximately the same yields were eventually obtained on mixtures with lactate. In the case of 1 per cent glucose, the addition of lactate may give significant increases. Mean values for the total yields of four sets of strain no. 97 measured after 600 to 800 hours and the results obtained with a set of strain no. 322 after 142 hours are shown in table 3. Many factors affect the final yields. Most important of these is, perhaps, the different extents of fermentation versus assimilation in growth of the available substrate occurring in the individual cultures. Furthermore, the long growth periods and different growth rates introduce variable changes in the medium leading to modifications in yields, partial autolysis, the presence of ghost cells, and perhaps even reutilization of substrate by new types. These difficulties do not arise with the glucose-adapted strain, where the relation of final yield to glucose content is very definite except insofar as the presence of 2 per cent lactate inhibits the growth on glucose. The cultures were not kept long enough to determine whether this lag in yields on 2 per cent lactate was ultimately recovered.

TABLE 3

Relative total yields

ORGANISM	MEDIUM							
	G_4 L_2	G_4 L_1	G_4 $L_{\frac{1}{2}}$	G_4	G_1 L_2	G_1 L_1	G_1 $L_{\frac{1}{2}}$	G_1
97	390	333	327	339	236	302	325	222
322	355	470	455	480	160	310	320	310

Final photometer readings: for strain no. 97, mean values for four sets after 600 to 800 hours' incubation, and for a single set of strain no. 322 after 142 hours.

To summarize, inoculation in glucose or glucose lactate mixtures does not result in immediate growth, as inoculation from lactate to lactate does. The delay is the incubation period required to adapt to the utilization of glucose. The incubation period may be shortened by additions of lactate, but when lactate is present the total growth and the growth rate are generally less and soon suffer a temporary stoppage. After this stoppage a secondary increase in growth occurs with a correspondingly slower rate of utilization of the remaining glucose and lactate.

Growth and Adaptation on Various Substrates

Adaptation to glucose on lactate medium. The foregoing results show that adaptation to glucose utilization occurs on synthetic medium with added glucose. It may also occur after very long periods on lactate media without added glucose. Thus, of some 50 cultures which have been tested for glucose adaptation after 600 or more hours of incubation on 1 and on ½ per cent lactate media, 3 and possibly several others were adapted to at least slow growth on glucose. It is not certain, however, that this adaptation has occurred in the absence of the substrate. More likely, some glucose has been produced in the cells as a result of hydrolysis of trehalose and perhaps of other polysaccharides, so that a condition comparable with that of added glucose obtains in old cultures of the unadapted strain.

Adaptation on standard peptone glucose broth. It was found that transfers of culture no. 97 from lactate to standard glucose broth containing yeast extract and peptone grew rapidly and, as a rule, exhibited vigorous fermentation within 24 to 48 hours' incubation at 30 C. This rapid adaptation could be due either to a specific factor in the natural medium or simply to the inclusion of different substrates which permit rapid multiplication of the unadapted cells, thus providing a large population for the selection of mutants.

Adaptation to fructose and sucrose, separately and in mixtures with lactate. Tests have been made of the ability of strain no. 97 to grow in synthetic media containing fructose, sucrose, or citrate and in mixtures of these sugars with lactate. The results presented in tables 4 and 5 are preliminary, but indicate that in general terms the behavior of the organism on fructose and sucrose is analogous to that on glucose. Adaptation occurs only after prolonged periods of incubation. Citrate is not a favorable substrate. The results recorded in table 5 show the effect of preliminary growth on one sugar medium on the subsequent growth on another substrate. Individual cultures designated in table 4 were transferred to three new media with lactate, glucose, or fructose (or sucrose) and the growth was observed. It may be seen that the behaviors of cultures from duplicate mother cultures are in good agreement. Such differences as exist in the time required for starting of growth are to be expected from the results given above and are no doubt, in part, in accordance with the extent of adaptation or rate of growth of the mother cultures at the time of transferring. The transfers from media containing only lactate to media containing only glucose, or fructose, do not start to grow in the 162 hours over which observations were made. On the other hand, all cultures grow on lactate, but the rate, especially at first, is slower for cultures derived from media containing one of the sugars alone than from those containing lactate or mixtures of one of the sugars with lactate. This confirms the indication given by the growth curves above that there may be a loss or regression in the capacity to grow on lactate that is associated with the acquirement of adaptation to a sugar.

Additional data included in table 4 show that at best only slight growth is obtained on the synthetic medium alone or with the amino acid mixture added.

Crosses and Backcrosses with Strain No. 97

Several crosses of the unadapted strain no. 97 with glucose-utilizing yeasts have been made by Mrs. Lindegren. Only a few ascospores were obtained in each successful case. The ascospores were isolated on a lactate medium like

TABLE 4

Relative growth of strain no. 97 on various substrates

CULT. NO.	SUBSTRATES ADDED	INCUBATION TIME IN HOURS				
		24	120	190	210	355
1	Fructose	—	—	g	+, f	
2		—	—	g	+, f	
3	4%	—	—	g	+, f	
4		—	—	g	+, f	
5	Fructose	tr	tr	g	+	
6	4%	tr	+, f	+	+	
7	Glucose	tr	tr	+	+	
8	2%	tr	tr	g	+	
9	Fructose	tr	tr	g	+	
10	4%	tr	tr	g	+	
11	Lactate	tr	tr	+	+	
12	1%	tr	tr	+	+	
13	Lactate	g	+	+	+	
14		g	+	+	+	
15	1%	g	+	+	+	
16		g	+	+	+	
17	Sucrose	—	—	g	+	+, f
18		—	—	g	+	+, f
19	2%	—	—	g	+	+, f
20		—	—	g	+	+, f
21	Sucrose	tr	tr	tr	g	+
22	2%	tr	tr	tr	g	+
23	Lactate	tr	tr	tr	g	+
24	1%	tr	tr	tr	+	+
25	Lactate 1%	g	g	+		
26	Am. Ac.	g	+	+		
27	Citrate 1%	tr	tr	g	g	g
28		tr	tr	g	g	g
29	Citrate 1%	tr	tr	g	g	g
30	Am. Ac.	tr	tr	g	g	g
31	Glucose	tr	tr	g	+	
32	2%	tr	tr	+	+	
33	Glucose 2%	tr	tr	tr	tr	tr
34	Am. Ac.	tr	tr	g	g	g
35	None	—	—	—	tr	tr
36		—	—	—	tr	tr
37	Amino	tr	tr	g	g	g
38	Acids	—	tr	g	g	g

Explanation of symbols: —, no growth; tr, trace of growth; g, definite growth; +, marked or nearly complete utilization of substrate; f, vigorous CO_2 evolution from fermentation.

Amino acid mixture (Am. Ac.) = glutamic acid 2.0 mg, arginine 2.0 mg, leucine 2 mg, per 10 ml of medium.

TABLE 5

Relative growth of transfers of strain no. 97 from media designated in table 4 on lactate, glucose, and fructose or sucrose

ORIGINAL		NEW SUBSTRATE	INCUBATION TIME IN HOURS		
Cult. no.	Substrate		18	120	162
1	F_4	L_1	tr	g	g
		G_2	—	g	+
		F_4	—	+, f	+
3	F_4	L_1	tr	+	+
		G_2	tr	tr	+
		F_4	—	g	+, f
9	F_4L_1	L_1	tr	+	+
		G_2	tr	tr	+
		F_4	tr	+, f	+, f
11	F_4L_1	L_1	g	+	+
		G_2	—	—	+
		F_4	—	g	+, f
13	L_1	L_1	g	+	+
		G_2	—	—	—
		F_4	—	—	—
15	L_1	L_1	g	+	+
		G_2	—	—	—
		F_4	—	—	—
31	G_2	L_1	tr	+	+
		G_2	tr	+	+, f
		F_4	—	g	+, f
32	G_2	L_1	tr	tr	tr
		G_2	tr	+	+, f
		F_4	—	g	+, f
17	S_2	L_1	—	g	g
		G_2	—	+	+
		S_2	tr	+, f	+
19	S_2	L_1	g	+	+
		G_2	+, f	+, f	+
		S_2	+	+, f	+
21	S_2L_1	L_1	tr	tr	g
		G_2	tr	+	+
		S_2	tr	+, f	+
23	S_2L_1	L_1	—	g	+
		G_2	—	—	—
		S_2	—	—	—

Symbols as in table 4. Incubation time on former medium 212 hours.

that described above, but matings were made in standard peptone glucose broth and the natural presporulation medium was used to induce sporulation. No clear segregation of the progeny has been found. In cases where all four spores from an ascus were viable, two often grew rapidly while two grew slowly, but when tested later for glucose utilization all would be positive. Viable progeny from backcrosses of these F_1 cultures with strain no. 97 were similarly positive. It is clear, however, from the evidence obtained later that rapid reversion to glucose utilization occurs on standard glucose broth and that such reversions would take place on the complex medium required for sexual fusion (Lindegren, 1945). The progeny not only may have been quickly adapted but may have been derived in the first place only from glucose-adapted parent cells.

DISCUSSION OF THE MECHANISM OF ADAPTATION

The present results do not permit an evaluation of the mechanism whereby adaptation to glucose utilization occurs. They fit the widely held view that adaptation occurs as a result of the occasional production of new types (mutants) from the unadapted population, but they do not exclude the opposite view of training of the existing cells. Even in the former case, it seems likely that not only the multiplication of the mutants but actually the mother cells are influenced by the presence of the substrate, so that the mutant does not arise merely by chance.

The data indicate that the reversion to glucose utilization is not a single step change occurring uniformly in individual cultures to restore the mutant to the original wild type. This suggests that the strain incapable of utilizing glucose does not differ by a specific single step change in the metabolic apparatus from the wild type.

Referring once more to the growth curves of cultures on mixtures of glucose and lactate, we note the high degree of variability, first, with respect to the rates of growth immediately after the beginning of measurable growth and, second, with respect to the lengths of time required from the beginning of adaptation to the attainment of the flex points. Inspection of the data on the growth of loop transfers from the main culture tubes to synthetic medium with glucose, which were made periodically to check the occurrence of adaptation to glucose, shows the following: In cases in which the rates of growth increase very rapidly from the start, the loop transfers grow immediately and vigorous fermentation sets in within 24 to 48 hours after inoculation. In cases in which the rates of increase are slow in the main tubes, loop transfers made even after considerable yields have been attained (up to one-third of the total yields) grow, at most, at slow rates on glucose for the first few days (often much slower than replicate transfers to lactate medium) and then show a rapid increase and fermentation. These results are interpreted to mean that at least two types of adaptation occur. There is adaptation to utilization of glucose in respiration and fermentation, and there is a development of resistance or tolerance to the presence of glucose in the medium. The two adaptations may not occur together or in this order.

It might be inferred, furthermore, from the variability in the slopes of the growth curves after adaptation to glucose, that the reversal to glucose utilization occurs by different paths, or more likely by different quantitative degrees, in individual cultures, i.e., the adaptation seems to involve multiple units, whether these be of the same or of different kinds. Conclusive proof as to which of these alternatives holds true will require many more quantitative measurements of growth rates than are now available. A similar difference in the degree of substrate utilization by a series of mutants has been reported by Zamenhof (1946) in the case of citrate utilization by mutants from a citrate-negative culture of *Escherichia coli.*

In the growth of a substrate-negative strain and its adapted progeny in a mixed medium containing the specific substrate, relationships are recognized to exist between (1) each organism and the components of the medium which it is specifically able to utilize, (2) competition of the two organisms for common substrates, and (3) less well-defined effects of metabolites of one organism on the growth of the other. The present results, if generally applicable, indicate that in addition there is a very marked effect on growth arising from relationships existing between each organism and component substrates which it is unable, though potentially able, to utilize. As far as we know, the latter effect has been neglected in kinetic treatments designed to distinguish between the different possible modes of origin of substrate-negative and substrate-adapted strains of microorganisms.

SUMMARY

Twelve strains of *Saccharomyces cerevisiae* have been isolated which were incapable of utilizing glucose for growth on an otherwise complete synthetic medium.

Subcultures from all but two quickly reverted to glucose utilization.

One of the latter two strains has been maintained glucose-negative for a period of 3 months in subcultures on the synthetic medium with lactate substituted for glucose as a carbon source.

This yeast is capable of reversion to glucose utilization (1) after prolonged incubation with glucose or mixtures of lactate and glucose present in the medium; (2) after exposure to standard glucose broth containing yeast extract and peptone; (3) after long periods of growth on the synthetic medium with low lactate content, under which conditions glucose is presumably formed.

Growth measurements indicate that the reversion to glucose adaptation involves more than a single step change, and occurs to quantitatively different degrees in different cultures.

Glucose markedly represses the growth of the glucose-negative cells, and, vice versa, lactate to some extent inhibits the growth of the adapted cells in cultures containing both substrates.

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THE EFFECT OF LARGE AMOUNTS OF NICOTINIC ACID AND NICOTINAMIDE ON BACTERIAL GROWTH[1, 2]

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This report deals with vitamin concentrations quite different from those usually employed. Previous work from many sources has given a reasonably accurate idea of the amounts of different vitamins needed by various microorganisms to support optimum growth. In contrast, little is known of the effect of high concentrations of vitamins. Evidence of inhibition or of harmful manifestations caused by large amounts of a vitamin have been infrequently reported for either microorganisms or higher forms.[3]

With respect to nicotinic acid and microorganisms the few available reports indicate that rather large amounts may be tolerated by several microorganisms without any obvious effect, but eventually a concentration is reached which causes inhibition. In a study of several strains of dysentery bacilli amounts of nicotinic acid (neutralized) up to and including 1,000 μg per ml in an amino acid, glucose medium produced no really marked retardation of growth, but concentrations of 3,000 and 5,000 μg per ml caused definite retardation, and 10,000 μg frequently produced complete inhibition (Dorfman, Koser, Reames, Swingle, and Saunders, 1939).

For *Proteus vulgaris*, Möller and Birkofer (1942) reported that 0.2 to 0.26 molar concentrations of nicotinic acid caused either reduction to half maximum growth or complete inhibition. The concentrations of nicotinamide causing similar effects were approximately 0.05 to 0.066 molar, amounts somewhat less than for the acid but still very high concentrations of vitamin. They also studied *Streptobacterium plantarum*, for which the figures were of about the same order of magnitude. These amounts are equivalent to approximately 6,000 to 30,000 μg per ml of medium. In their study growth appears to have been recorded at only 24 and 48 hours, in some cases only the former. If delayed growth had been taken into account in their determination of inhibition, it is quite possible their results might be more nearly in agreement with those of the dysentery bacilli previously reported.

The inhibition caused by large amounts of nicotinic acid was evident also in a study of nicotinic acid destruction (Koser and Baird, 1944) in which the vitamin was supplied as the only organic compound (foodstuff and vitamin) to a few cultures able to grow under these conditions, *Pseudomonas fluorescens*, *Serratia*

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[1] This work was aided by a grant from the United States Public Health Service.

[2] Presented, in part, at the 47th General Meeting of the Society of American Bacteriologists, Philadelphia, Pennsylvania, May, 1947.

[3] A summary of the information pertaining to experimental animals and man has recently appeared (Nutrition Reviews, 1947).

marcescens and allied types. These organisms often were either retarded in their growth or completely inhibited by 0.5 and 1.0 per cent concentrations of nicotinic acid (5,000 and 10,000 μg per ml) though they were able to grow in the presence of, and break down, smaller amounts of vitamin.

It appears, however, that other groups of microorganisms may be affected at quite different levels. In a study of *Leptospira canicola*, Rosenfeld and Greene (1941) found that 10 μg of nicotinic acid or amide caused distinctly poorer growth than 1.0 μg per ml.

In the present investigation a number of the commoner bacteria were employed. It was desired to use a simple synthetic medium for much of the work, and hence the experiments usually were limited to organisms which would develop in such a medium, either with or without the addition of known vitamins. Certain species were chosen because of a need for the vitamin, nicotinic acid, or nicotinamide, which was to be used in large amounts; others because they do not need this compound or do not need any preformed vitamins.

METHODS

Basal medium. The following medium was used for most of the experiments:

$(NH_4)_2HPO_4$	2.0 g
KH_2PO_4	1.5 g
NaCl	5.0 g
$MgSO_4$	0.1 g
Glucose	5.0 g
Redistilled water	1,000 ml

The pH of the medium is 6.8 to 6.9, but requires readjustment to this figure after the addition of large amounts of nicotinic acid. Glucose was dissolved separately in redistilled water to make a 25 per cent solution, sterilized either by sintered glass filtration or the autoclave, and added to the previously tubed medium to give 0.5 per cent concentration.

For some organisms it was necessary to add a few amino acids or vitamins, or both, to secure prompt and satisfactory growth for the tests. Pelczar and Porter (1943) reported that 0.024 mg of cystine per ml is sufficient for *Proteus morganii*. Cystine and glutamic acid in amounts of 0.024 mg and 1.0 mg per ml of medium, respectively, were added for the cultivation of *P. vulgaris*, *P. morganii*, and representatives of the *Bacillus* genus. Cystine, glycine, and arginine in amounts of 0.024, 0.75, and 3.8 mg per ml, respectively, were used for two strains of *Staphylococcus aureus*. For these two cultures and for information concerning their amino acid needs we are indebted to Dr. Hite of this laboratory (Surgalla and Hite, 1946). In addition to nicotinic acid or nicotinamide, which was added in large amounts, 0.2 μg of calcium pantothenate per ml was used for *P. morganii* and 0.1 μg thiamine and 0.01 μg biotin per ml of medium were used for *S. aureus*. The medium was distributed into 16-by-150-mm test tubes previously standardized for use in the colorimeter. The total volume of medium was 5.0 ml per tube.

Inoculum. From 24-hour agar slants, suspensions were prepared in sterile, phosphate-buffered sodium chloride solution. The amount of culture carried over was sufficiently small so that barely perceptible turbidity in the salt solution could be discerned in a good light. One-tenth ml of this suspension was then used to inoculate each tube of a series in the test. Agar plate counts showed that such an inoculum usually contributed from 60,000 to 130,000 cells per ml of medium. This is not a large inoculum, but it is sufficient to produce marked turbidity in the synthetic medium within 16 hours after inoculation with most of the cultures. To test the possible inhibitory effect of high concentrations of vitamin, it was desired to have an inoculum capable of giving rise to distinct growth in the control tubes within 16 to 24 hours.

Measurement of growth. Estimations of comparative growth were made on the basis of turbidity, which was measured by means of an Evelyn type photoelectric colorimeter. All cultures were incubated at 37 C with the exception of *Pseudomonas fluorescens*, *Agrobacterium tumefaciens*, *Serratia marcescens*, and the sporeforming bacilli, which were held at 30 C.

EXPERIMENTAL RESULTS

Preliminary tests showed that amounts of nicotinic acid up to 1,000 μg per ml caused no marked inhibition of growth of some of the commoner bacteria. Tests were then made using increasing amounts of both nicotinic acid and nicotinamide up to 10,000 μg per ml. In each series of tests, tubes containing 1.0 μg of vitamin per ml were always included. These received the same amount of inoculum and served as a basis of comparison for the larger amounts. The 1.0-μg amount was used as a routine in all tests, whether or not the organism in question needed the preformed vitamin for prompt growth.

Typical results are shown in table 1. The general picture is one of increasingly retarded growth as the concentration of vitamin is raised from 1,000 to 10,000 μg per ml. In the presence of 1,000 μg, slightly retarded growth, compared with the 1.0-μg level, was observed with about half of the cultures, particularly at the earlier periods of observation. At times this retardation persisted throughout the entire period of incubation, growth never quite equaling that in the 1.0-μg tubes, but more commonly the initial differences between 1.0 and 1,000 μg tended to become insignificant or to disappear entirely during the later stages of growth. At 3,000 μg retardation of growth usually was distinct and definite at first, but somewhat less definite later; 5,000 and 10,000 μg produced more marked retardation or complete inhibition of growth.

Several repetitions of the tests shown in table 1 confirmed the general picture of retarded growth and occasional complete inhibition. Although there were some differences in detailed results from one test to another, usually such differences were within limits that might be expected. Many of these minor differences appeared in the 10,000-μg tubes, where growth either was scanty and slow on one occasion or failed completely to appear in another test. For example, *Shigella paradysenteriae* Strong 3, which grew poorly in the presence of 10,000 μg nicotinic acid in the test shown in table 1, in other tests failed at times to grow in this concentration of vitamin. The Sonne type 269 (not shown in the table) occasionally grew slowly in the 10,000-μg tubes, though the majority of

TABLE 1

The effect on growth of massive doses of nicotinic acid and nicotinamide in a synthetic medium

ORGANISM	CONC. OF VITAMIN	NICOTINIC ACID				NICOTINAMIDE			
		Growth at hours				Growth at hours			
		16	24	48	96	16	24	48	96
	µg/ml								
Shigella paradysenteriae	1	24	41	49	50	23	42	47	50
	1,000	17	34	31	43	14	32	40	48
	3,000	4	16	24	28	2	24	35	41
Strong 3	5,000	1	17	25	24	0	5	31	28
	10,000*	0	0	12	12	0	0	0	0
Shigella paradysenteriae	1	14	35	37	40	12	37	39	42
	1,000	9	26	24	31	10	35	38	42
	3,000	2	13	16	16	1	10	26	24
Flexner W	5,000	0	5	10	9	0	2	8	21
	10,000	0	0	0	1	0	0	0	0
Shigella alkalescens	1	40	38	43	58	40	39	43	55
	1,000	31	32	33	45	37	38	41	56
	3,000	23	28	30	36	33	36	39	52
	5,000	19	24	25	27	21	36	38	44
	10,000	11	20	22	26	0	0	8	27
Escherichia coli 1	1	40	42	48	53	40	39	46	51
	1,000	31	35	38	43	38	37	44	46
	3,000	27	29	32	36	32	39	47	51
	5,000	21	24	25	30	21	35	40	45
	10,000	10	17	18	21	0	0	0	1
Salmonella schottmülleri	1	38	41	51	55	36	39	50	55
12	1,000	18	42	47	54	18	39	50	55
	3,000	3	23	40	47	2	12	47	55
	5,000	1	6	28	35	0	3	43	53
	10,000*	0	0	5	15	0	0	0	41
Proteus vulgaris Y	1	19	39	47	52	31	37	47	50
	1,000	12	39	46	52	20	36	47	49
	3,000	4	29	42	50	13	36	48	49
	5,000	0	7	34	39	2	5	34	41
	10,000	0	0	0	0	0	0	0	0

Of the cultures listed above, the dysentery bacilli, *P. vulgaris*, *P. morganii*, and *S. aureus* need nicotinic acid for growth. The remaining cultures grow in the basal medium without the addition of preformed vitamins. Other vitamins and amino acids needed by *P. morganii* and *S. aureus* were supplied in amounts sufficient for optimum growth.

The figures for growth represent turbidity on a scale of 0 for a clear tube and 100 for no light transmission.

* The results were somewhat variable in repeated tests, showing either no growth or light delayed growth.

TABLE 1—*Concluded*

ORGANISM	CONC. OF VITAMIN	NICOTINIC ACID				NICOTINAMIDE			
		Growth at hours				Growth at hours			
		16	24	48	96	16	24	48	96
	µg/ml								
Proteus morganii 18	1	14	45	51	56	15	42	48	53
	1,000	7	38	46	51	9	27	46	51
	3,000	3	15	36	39	3	10	37	42
	5,000	0	5	31	32	1	4	34	38
	10,000	0	0	16	25	0	0	3	6
Pseudomonas fluorescens 12	1	3	14	64		2	14	65	
	1,000	1	17	52		1	8	44	
	3,000	2	10	37	47	0	5	41	
	5,000	0	3	34	42	0	0	20	42
	10,000	0	0	7	24	0	0	0	0
Agrobacterium tumefaciens	1	4	10	20	39	5	11	21	39
	1,000	4	10	18	31	4	10	17	32
	3,000	0	6	13	20	2	9	13	18
	5,000	0	4	11	18	0	6	11	19
	10,000*	0	0	0	2	0	0	1	10
Staphylococcus aureus 161	1	2	20	44	45	0	21	43	44
	1,000	2	18	34	41	0	12	38	38
	3,000	1	10	24	29	0	10	33	40
	5,000	0	6	15	20	0	4	19	28
	10,000	0	2	4	17	0	2	2	4
Bacillus subtilis	1	0	2	13	20	0	2	17	26
	1,000	0	5	17	22	0	3	16	23
	3,000	0	0	10	16	0	2	17	23
	5,000	0	0	3	8	0	0	14	24
	10,000	0	0	0	4	0	0	10	30
Bacillus megatherium	1	1	7	19	36	0	3	15	33
	1,000*	0	0	0	0	0	0	0	0
	3,000*	0	0	0	0	0	0	0	0
	5,000	0	0	0	0	0	0	0	0
	10,000	0	0	0	0	0	0	0	0

tests were negative at this level. Similar minor differences in results were seen, particularly in the case of *Salmonella schottmülleri*, *A. tumefaciens*, and *Bacillus megatherium.* The strain of *B. megatherium* used in this study was more sensitive to the effect of increased amounts of vitamin than the other cultures, though here again some differences in the results of repeated tests were seen. In one case, *B. megatherium* grew slowly and poorly in the presence of 1,000 and 3,000 µg per ml of nicotinic acid or nicotinamide; in other tests it failed to grow in the presence of 1,000 µg and larger amounts of vitamin.

Some additional cultures, not shown in table 1, were used also. Other representative strains of dysentery bacilli, a strain of *Escherichia coli*, one of *P. fluorescens*, and several strains of *Salmonella aertrycke* and *Salmonella enteritidis*, were affected in essentially the same way as those shown. Another strain of *Proteus vulgaris* proved to be somewhat less sensitive to the inhibitive effects of large amounts of vitamin than the Y strain of table 1; it was able to grow regularly, though slowly, in the presence of 10,000 μg of either nicotinic acid or amide. An additional *P. morganii* behaved similarly to the number 10 given in the table. A laboratory stock culture labeled *Bacillus vulgatus* responded to increased amounts of vitamin much as did *Bacillus subtilis*.

In some of the tests the nicotinic acid or amide was added to the medium before sterilization and was autoclaved in the medium; in other experiments this procedure was varied to avoid heating the vitamin in the medium. A neutralized solution of vitamin was either filtered separately through sintered glass filters and added to the previously autoclaved medium, or the vitamin was incorporated directly, in the desired concentration, in the unsterilized synthetic medium and the completed medium then sterilized by filtration and transferred aseptically into tubes. The results of comparative tests with a number of cultures showed no real difference in the effect produced by the 1,000-, 3,000-, 5,000-, and 10,000-μg concentrations, whether the vitamin was filtered or autoclaved in the medium.

The tests shown in table 1 were made with commercial samples of the vitamin. Although the product is known to be one of high purity, it was desired to check the results with a specially purified sample. Accordingly a small quantity of nicotinic acid was sublimed and recrystallized from water.[4] Comparative tests were made with the sublimed and the commercial product, using representative cultures from among those shown in table 1. The results were the same in each case: the degree of inhibition caused by the 3,000, 5,000, and 10,000 concentrations was of the same order of magnitude. There seems to be no reason for believing that the inhibitive effects of nicotinic acid were caused by an impurity in the sample.

On substitution of 0.5 per cent glycerol for glucose in the synthetic medium, growth of the bacteria was slower, requiring often several days to reach fullest development; but the inhibitive effect of the 3,000-, 5,000-, and 10,000-μg concentrations of both nicotinic acid and nicotinamide was just as apparent as in the presence of glucose.

Successive transfers. Four cultures which were inhibited by 10,000 μg of the vitamin were grown successfully in the basal synthetic medium, with 0.5 per cent glucose, through 35 transfers in the presence of 5,000 μg nicotinic acid per ml. Growth appeared rather readily on successive transfers, often within 24 hours. On microscopic examination the cells appeared somewhat swollen and stained rather irregularly with fuchsin or gentian violet, in contrast to their usual appearance on the synthetic medium with smaller amounts of vitamin or on agar slants.

Casein digest. When acid-hydrolyzed casein digest supplemented with 24 μg cystine per ml was substituted for the ammonium nitrogen of the basal synthetic medium, the retarding effect of high vitamin concentrations was distinctly less pronounced. The 3,000-μg level caused slight growth retardation, which often was evident only during the earlier periods of observation. Five thousand μg produced a somewhat more marked effect, though here, too, growth at times approached that in the 1.0-μg controls after 40 to 48 hours' incubation. At 10,000 μg, growth was retarded, though seldom completely inhibited. The results with two cultures, which are representative of 12 others compared in this way, are shown in figure 1.

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[4] The writers are indebted to Dr. J. W. Moulder of this department for the sample of sublimed nicotinic acid.

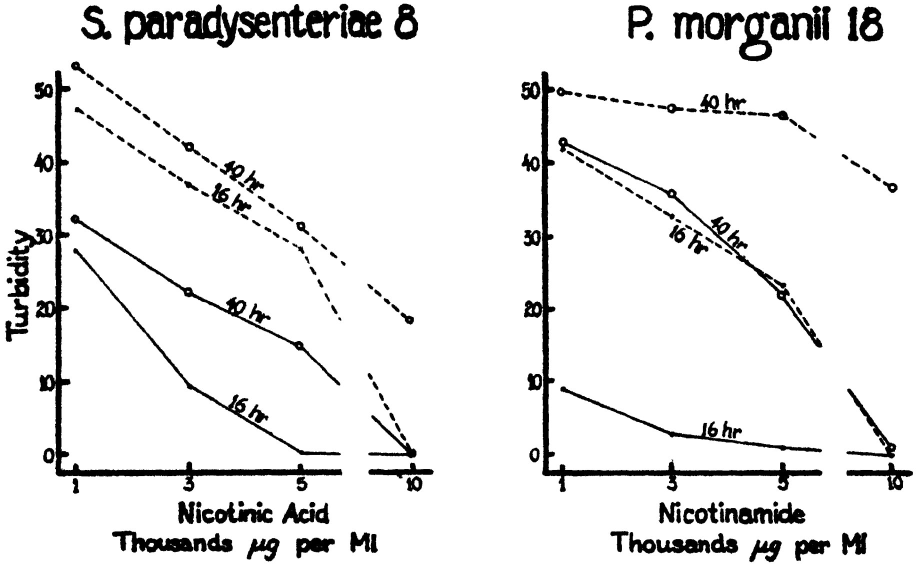

Fig. 1. Growth Response of Two Cultures in the Ammonium Salt Synthetic Medium and in Casein Digest Medium in the Presence of High Concentrations of Vitamin

———, ammonium salt medium.

- - - - - -, casein digest medium.

● = 16-hour, and ○ = 40-hour, results.

Turbidity of the cultures, as shown by colorimeter readings, is plotted against the micrograms of vitamin per ml of medium.

Evidently, with a varied supply of amino acids at hand the effect of vitamin imbalance is less marked. A comparable effect was not secured by increasing the amount of inorganic nitrogen in the ammonium phosphate synthetic medium, an experiment which was tried with several levels of the nitrogen compound but with results quite different from those secured with the casein digest.

Yeast extract. The inhibition ordinarily produced by 10,000 μg per ml of nicotinic acid or amide was nullified in the presence of comparatively small amounts of yeast extract. Typical results are given in table 2, which summarizes experiments in which decreasing amounts of yeast extract were added together with 10,000 μg of vitamin and the resultant growth was compared with that in the basal medium plus 1.0 μg of vitamin. One-tenth per cent yeast extract, or

1,000 μg of dry material per ml, nullified the inhibition quite effectively. With decreasing amounts of yeast extract this effect became less apparent, until at levels of 0.001 to 0.0001 per cent, or 10 and 1.0 μg of dry yeast extract per ml, the "protective" effect of the yeast extract was no longer evident. Similar results were secured with several other cultures.

Known compounds. The effect of yeast extract could not be duplicated with a mixture of known vitamins and compounds of related interest. At best only a rather feeble countering of the effect of 10,000 μg nicotinic acid or amide was secured by a mixture of twelve crystalline vitamins, purines, and other substances. In table 3 this is seen in the case of the Sonne dysentery number 8 culture, in which light delayed growth appeared in comparison with the clear tubes of the 10,000-μg amount without the added growth factors. Even this slightly beneficial effect of the growth factors was not always apparent. The growth of *P. vulgaris* Y was not aided by the mixture of vitamins in the presence of 10,000 μg nicotinic acid or amide. However, several other cultures, including three additional strains of dysentery bacilli and another of *P. vulgaris* (not shown in the table), gave results more in line with those of the Sonne 8 culture.

TABLE 2

The effect of varying quantities of yeast extract in the presence of large amounts of vitamin

AMMONIUM PHOSPHATE SYNTHETIC MEDIUM PLUS		SHIGELLA PARADYSENTERIAE SONNE 8							
		Nicotinic acid				Nicotinamide			
Vitamin	Yeast extract	Growth at hours				Growth at hours			
		16	24	48	96	16	24	48	96
μg/ml	%								
1.0		32	41	48	50	33	41	47	50
10,000		0	0	0	0	0	0	0	0
10,000	0.1	26	30	32	40	40	41	45	51
10,000	0.05	23	24	26	34	37	39	43	48
10,000	0.01	13	15	16	17	19	22	22	24
10,000	0.005	9	10	11	11	12	19	21	21
10,000	0.001	5	7	9	12	0	0	2	2
10,000	0.0005	4	4	7	9	0	0	0	0
10,000	0.0001	1	2	2	2	0	0	0	0

The figures for growth represent turbidity on a scale of 0 for a clear tube and 100 for no light transmission.

An increase in the amount of vitamins and other compounds in the mixture to from 5 to 100 times the concentrations first used produced little improvement in overcoming the inhibition caused by the 10,000 μg of nicotinic acid. In no case did the effect of the vitamin mixtures approach that of 0.1 per cent yeast extract, though the total dry weight of crystalline substances added was substantially the same as the amount of yeast extract. The vitamin mixture and casein digest, when added together, were as effective as yeast extract in counteracting the inhibition of 10,000 μg of nicotinamide in the case of the Sonne 8 culture (table 3).

A few crystalline compounds were added singly in the presence of large amounts of nicotinic acid or amide, but at best they exerted little effect in overcoming the inhibition caused by 10,000 μg of the vitamin. The list comprised several amino acids including cystine, thioglycolate, methionine, and choline.

Higher amounts of nicotinamide. Since a number of the bacteria grew readily in the presence of 10,000 μg per ml of nicotinic acid or amide when yeast extract was supplied, several higher amounts of vitamin were tried in the presence of yeast extract. Of 16 bacteria tested, only the staphylococci were able to grow under such conditions. Two strains of *S. aureus* and one of *Staphylococcus albus* produced visible growth within 24 or 48 hours, and moderate growth later, in the presence of 20,000 or 30,000 μg of nicotinamide and 0.1 per cent yeast extract. All other types failed to grow in the presence of 20,000 μg nicotinamide, though they developed readily in 10,000 μg of the vitamin with 0.1 per cent yeast extract.

TABLE 3

The effect of yeast extract, crystalline vitamins, and casein hydrolyzate in the presence of large amounts of vitamin

AMMONIUM PHOSPHATE SYNTHETIC MEDIUM, PLUS				S PARADYSENTERIAE SONNE 8					P. VULGARIS Y				
Nicotinic acid or amide	Yeast extr.	Vit. mixt	Casein hyd.	Growth at hours					Growth at hours				
				16	24	40	48	96	16	24	40	48	96
μg/ml	%		%										
Na 10,000				0	0	0	0	8	0	0	0	0	0
Na 10,000	0.1			31	34	36	36	42	18	35	38	39	44
Na 10,000		+		2	9	11	11	12	0	0	0	1	2
Nm 10,000				0	0	0	0	0	0	0	0	0	0
Nm 10,000	0.1			31	31		36	44	3	10		33	40
Nm 10,000		+		0	0		11	19	0	0		0	0
Nm 10,000			0.5	8	36		42	48	3	16		38	46
Nm 10,000		+	0.5	31	32		37	40	4	20		36	43

Na = nicotinic acid; Nm = nicotinamide.

The vitamin mixture contained thiamine, calcium pantothenate, pyridoxine, riboflavin, folic acid, *para*-aminobenzoic acid, biotin, adenine, guanine, choline, inositol, and glutamine, and was added in an amount to supply enough of each factor for optimum growth of organisms known to require these substances.

DISCUSSION

A comparison of the amount of nicotinic acid or amide needed for optimal growth with the amounts needed to produce an inhibiting effect shows a wide margin indeed. Less than 0.1 μg of nicotinamide is sufficient for optimal growth of the dysentery bacilli and *Proteus* under the conditions of these tests. Organisms such as *E. coli*, *Aerobacter aerogenes*, and related types, which do not need the preformed vitamin, have been reported to synthesize from about 0.3 to 1.0 μg of nicotinic acid per ml of culture (Burkholder and McVeigh, 1942; Perlman, 1945). Several thousand times or, indeed, many thousand times the amount needed for optimal growth can be supplied before a really distinct growth-retarding effect is observed. Whether the same finding might apply to groups of bacteria other than those used in this study is not known.

The experiments in which the synthetic medium was used represent an extreme example of nutritional imbalance, for here the cells were supplied massive doses of one vitamin in an environment in which other factors must be synthesized. Under such conditions the limiting effect of high concentrations of vitamin was more marked than when preformed compounds were supplied in casein hydrolyzate or in yeast extract.

There is evidently in yeast extract something quite effective in nullifying the inhibition caused by massive doses of nicotinic acid or nicotinamide, but this effect could not be duplicated with a mixture of known crystalline vitamins and related compounds. Whether the effect of yeast extract may reside in only one or a few unidentified substances or be due more to a varied assortment of preformed vitamins, amino acids, and other nutritive substances is not definitely known. The results with casein hydrolyzate and mixtures of known vitamins seem to point to the latter explanation as more nearly correct.

When an array of nutritive substances was supplied preformed, the organisms seemed to experience little difficulty in carrying on metabolism, as expressed by the growth of cultures, in the presence of such large amounts as 10,000 μg of one vitamin. When the nutritive environment was strictly limited, and many substances had to be synthesized by the cells, growth occurred but poorly or not at all in the same concentration of nicotinic acid. Stated differently, it might be said that in the presence of high concentrations of one vitamin the cells are more exacting and under such conditions fail to grow, or grow slowly, unless a varied assortment of other nutritive substances is supplied.

SUMMARY

An increase in the concentration of nicotinic acid or nicotinamide from the usual small amount, which supports optimum growth, to 1,000 μg per ml of medium produces relatively little effect upon the rate of growth of a number of representative bacteria.

Concentrations of 3,000 and 5,000 μg per ml retard growth more markedly, and 10,000 μg often inhibits it completely in a simplified culture medium. Results are affected by the basal medium used. In casein hydrolyzate the inhibition by large amounts of the vitamin is less marked.

Yeast extract nullifies the inhibiting effect of 10,000 μg nicotinic acid or nicotinamide, but a mixture of crystalline vitamins and related compounds is much less effective or is ineffective in replacing yeast extract.

The large amounts of vitamin in the synthetic medium represent an extreme example of nutritional imbalance, in which the cells are supplied enormous amounts of one vitamin in an environment in which many other nutritive substances must be synthesized. Such maladjustment can be carried to surprising levels before distinct retardation of cell metabolism, as expressed by multiplication, becomes apparent. In the presence of certain amounts of vitamin which

inhibit growth, bacteria which are not ordinarily exacting in their nutritive requirements appear to be more exacting, and often fail to grow unless other preformed substances in casein digest, and especially in yeast extract, are supplied.

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HEMOPHILIC GRAM-POSITIVE RODS

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A number of bacterial species are known to prefer media containing blood. An analysis of the growth requirements of such bacteria, however, in many cases shows that it is the protein contained in the blood which favors growth, and that this protein can be supplied in the form of any body fluid, such as ascites fluid, or by other proteins or protein derivatives. Strictly hemophilic species, in the sense that they require the X and V factors of Thjøtta and Avery, are not known to exist outside the genus *Hemophilus* To our knowledge no gram-positive organism is known to require these factors. If any such observation has been published, we have been unable to find it in the literature.

A few months ago one of us, while examining a blood agar culture, found some colonies which showed a marked satellite phenomenon, growing only in the neighborhood of other bacterial colonies, chiefly staphylococcus colonies, which grew in the same culture. The colonies resembled those of *Hemophilus influenzae*, but a microscopic examination showed that they actually contained gram-positive rods with the morphological characteristics of diphtheroids, such as *Corynebacterium pseudodiphthericum.*

It was intended to make a detailed study of this organism, but unfortunately it died before the study was complete. Some experiments were made, however, on the growth-promoting effect of various other bacteria. In these experiments the gram-positive rod was streaked on blood agar plates with a loop in parallel streaks, whereas the various other microbes to be tested were streaked at right angles to the gram-positive rod, in widely separated streaks.

Growth of colonies of the gram-positive rod was practically exclusively obtained close to the growth of the test organisms, where they were fairly large up to 1 mm in diameter. But at a distance from the test organism of ½ to 1 cm the growth of the gram-positive rod seemed to fail, and midway between two streaks of test organisms either no colonies at all could be seen, or only a few of pin-point size or smaller.

Experiments with various other microbes gave the following results: An optimal growth-promoting effect was obtained with *Staphylococcus aureus*, *Staphylococcus albus*, *Bacillus anthracis*, and *Bacillus subtilis*. A good effect was obtained with strains of *Alcaligenes*, *Escherichia*, *Klebsiella*, *Listerella*, *Neisseria pharyngis*, *Pseudomonas aeruginosa*, *Saccharomyces*, *Sarcina*, *Salmonella*, and *Streptococcus pyogenes*. A variable effect was obtained with different strains of diphtheroids, some showing slight activity, others being entirely ineffective. No effect at all was obtained with *H. influenzae* (see nos. 1 to 3 in figure 1).

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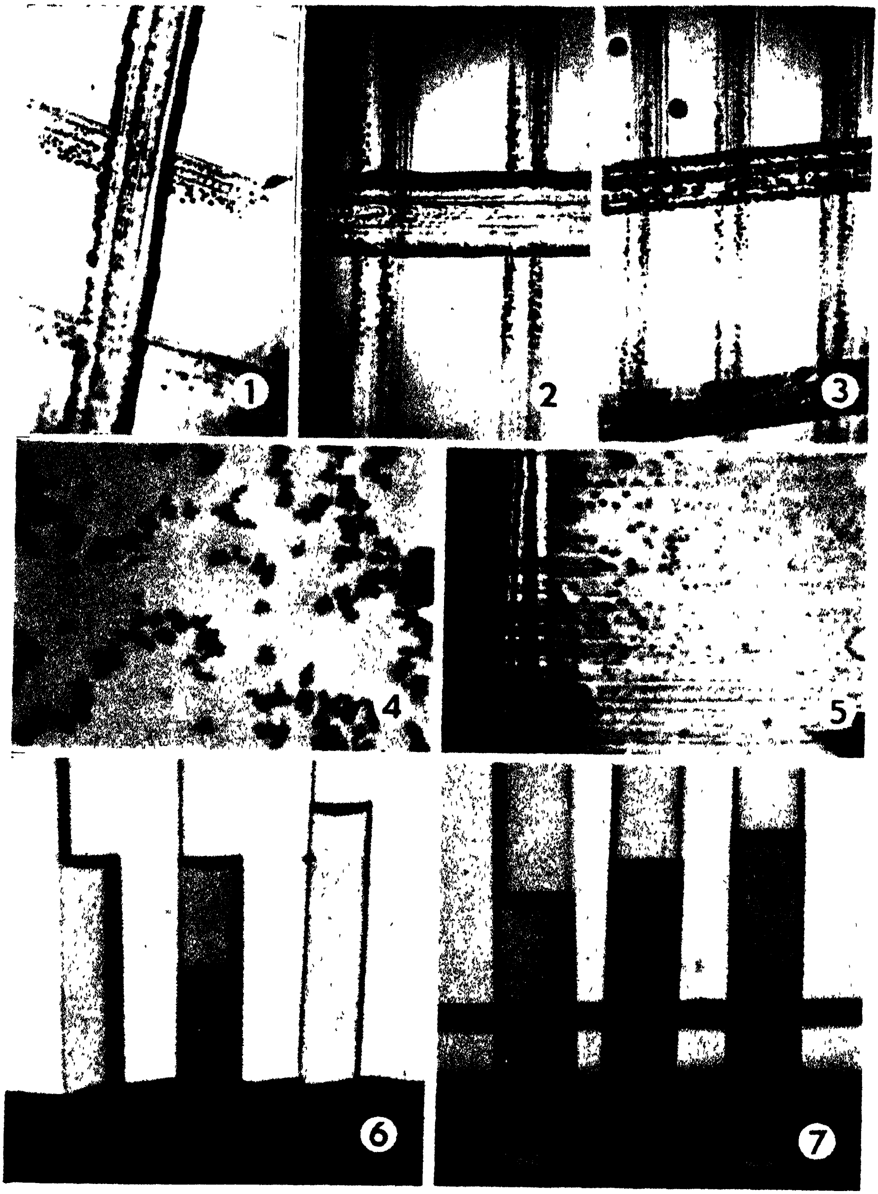

FIG. 1

No. 1. Strain no. 1 growing as a satellite to *Staphylococcus albus*. Blood agar. × 5.
No. 2. Same strain growing as a satellite to *Escherichia coli*. Blood agar. × 5.
No. 3. Same strain growing as a satellite to a viridans streptococcus. Blood agar. × 5.
No. 4. Strain no. 2. Gram-stained film. × 1,150.
No. 5. Strain no 2 growing as a satellite to *Staphylococcus aureus*. Blood agar. × 2.
No. 6 Strain no. 2. Growth in ascites broth with X factor (left tube, no growth), V factor (right tube, no growth), and X + V factor (middle tube, fairly heavy growth with particularly heavy turbidity in lower half of tube).
No. 7. Strain no. 2. Growth in ascites peptone water with 1 per cent glucose and Andrade's indicator. Left tube: with V factor (no growth, no fermentation). Right tube with X factor (no growth, no fermentation). Middle tube: with both X and V (good growth and acid production, shown by red color). Photographed with a green filter.

Thus a number of different organisms produced the factor which our strain required for its growth. By analogy, it seems likely that this must have been the V factor, as most of the same organisms show a growth-promoting effect on *H. influenzae* also. Proof can unfortunately not be offered so far as this strain is concerned.

In regard to other properties of this strain, it was found that it grew equally well aerobically and anaerobically, and that in some cultures it produced two different types of colonies, one round, smooth, glistening, and slightly raised, the other smaller, thinner, and slightly irregular. Both these colonies showed the satellite phenomenon, and the small irregular type seemed to revert to the smooth type in subculture.

Some time later another one of us became aware of some similar colonies in a blood agar culture from the pus of a purulent pericarditis. These colonies grew as satellites around colonies of *Staphylococcus aureus*. They were indistinguishable from those of *H. influenzae*, but were found to contain gram-positive rods of the same type as *C. pseudodiphthericum*. They had the properties which follow:

Morphology. Short gram-positive rods, measuring about 1 μ by 3 to 4 μ, straight, and with rounded ends. Some rods showed clublike swellings of one end, and they showed a marked tendency to parallel, "palisade" arrangement. They showed a great resemblance to *C. pseudodiphthericum* (figure 1, no. 4). The rods were nonmotile.

Colony. Small (up to about 1 mm), flat, transparent, colorless, smooth, and glistening, with a soft, homogeneous consistency. They resembled those of *H. influenzae.*

Growth requirements. No growth was obtained on media not containing blood or the X and V factors. On blood agar plates growth was practically exclusively obtained in the neighborhood of colonies of other organisms. Staphylococcus colonies had a particularly strong growth-promoting effect (figure 1, no. 5).

In fluid media no growth at all was obtained in peptone water or meat infusion broth, nor in the same media after addition of either the V or the X factor. Just perceptible growth was obtained in meat infusion broth containing both X and V factor (in the form of yeast extract and autoclaved blood extract). In ascites broth without X or V, or with only one of them, no growth occurred, but a heavy growth, with dense, even turbidity, was obtained in this medium with both factors present. Thus this strain is very exacting. It is unable to grow unless both X and V factors are present, and in addition it requires a medium rich in protein in order to make more than an exceedingly meager growth (figure 1, nos. 6 and 7).

Fermentation reactions. Twenty per cent ascites fluid and the X and V factors were added to peptone water tubes containing 1 per cent of various sugars, alcohols, or glucosides. Weak acid was produced from glucose and fructose only. In tubes sealed with vaseline no growth occurred, indicating that this strain was strictly aerobic.

DISCUSSION

The two strains of gram-positive rods described above showed unusual growth requirements. The first strain, though incompletely studied, obviously demanded some growth factor not present, or present in too small quantities, in blood agar. There is some circumstantial evidence that this factor was the V factor.

It is evident that the second strain had an absolute need for both the X and the V factors, since no growth was obtained without them, and only very poor growth on blood agar which contains enough of the X factor but which may be somewhat poor in V. It seems clear that the growth-promoting effect of other microbes must have been due to their production of the V factor. This second strain was even more exacting in its demands than *H. influenzae*, as it refused to produce more than a very minimal growth unless ascites fluid was also added to the medium.

Although we have no direct proof that our strains actually came from the material inoculated on the primary blood agar plates, it is felt that they must ultimately have come from an animal source, since it seems unlikely that such strains could exist except as parasites on some animal host.

The classification of the first strain is impossible owing to incomplete information, and that of the second strain is somewhat difficult in view of its unusual properties. It is known that many lactobacilli have peculiar nutritional demands, but it is not thought that our strain resembles the lactobacilli. It does not show the tendency to chain formation often seen in lactobacilli, but rather the palisade formation of the diphtheroids. Further, it is a weak acid producer, in contrast to the lactobacilli, but in fair agreement with many diphtheroids. To classify our strain as a *Hemophilus* species would be a mistake, since this genus should be reserved for gram-negative rods.

In our opinion this strain should be classified as a *Corynebacterium*. Its hemophilic properties might be used in coining a name, and the name *Corynebacterium hemophilum* is suggested in case future investigations should justify its rank as a species.

SUMMARY

Two strains of gram-positive rods are described, which grow mainly as satellites around other bacterial colonies on blood agar. Both strains required some growth factor produced by other bacteria in addition to the substances contained in blood agar.

The only strain studied in detail was absolutely dependent upon both the X and V factors of Thjøtta and Avery and, in addition, showed a great preference for media rich in animal protein.

It is suggested that this strain should be classified as a *Corynebacterium*, and, should future investigations justify it, under the specific name *C. hemophilum*.

CHEMICAL DETOXIFICATION OF FLEXNER DYSENTERY ANTIGEN

II. Studies of Accelerated Growth Rates[1]

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A major problem which arises in work on the chemistry of bacteria is that of obtaining adequate amounts of organisms with the facilities of the ordinary research laboratory. For this purpose, in growing bacteria for chemical studies on Flexner dysentery organisms (Barnes, Dewey, Henry, and Lupfer, in press), a method was developed for obtaining large amounts of this organism rapidly, and in high concentration, using simple constituents and standard laboratory apparatus. The method is based on the observations that the organism flourishes in the presence of aeration (Topley and Wilson, 1936; Hoberman and Dubos, unpublished observations), and that growth is enhanced if the effect of the hydrogen ions produced during growth is buffered by suitable salts (Hoberman and Dubos). Conditions for culturing organisms in which a large surface is exposed to a gas phase have been described by several investigators (Mironova, 1941; Clifton, 1943; Johnson, Bruce, and Dutcher, 1943; Hoberman and Dubos).

By this method the virulent type III (Z) strain with which we were concerned was grown to concentrations 8 to 10 times those obtained over 18 to 24 hours by usual culture methods (table 1, figure 1). Also, the rate of growth was increased so greatly that each liter of culture medium produced this concentration in 2½ to 3 hours. In this period, therefore, the final result was a net increase of 60- to 80-fold over the usual 20-hour growth rates. If, at the end of the 3-hour period, the bacteria were kept in the logarithmic phase of growth through the prompt addition of a fresh liter of medium, the effect could be reproduced as often as desired.

The logarithms of the values for turbidity and live count are plotted against time in figure 2. It may be seen from this figure that the rapid regeneration rate of the organisms in still media during the first hour was continued after shaking began. During the logarithmic phase the number of live organisms doubled every 40 minutes, but after about 2½ hours of shaking the regeneration rate decreased sharply. The relative increase in mass during the shaking period was only 3-fold, whereas that in numbers was 30-fold (table 1, figure 1). It is apparent that there is an extensive mean loss in mass for the individual organism during this period of rapid growth. However, the yield of antigen, as judged by the mouse test (Barnes and Dewey, to be published), was that expected from the increased numbers of organisms. Further study of this phenomenon is required to elucidate its place in bacterial growth.

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[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Children's Hospital, Cincinnati, Ohio.

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Both smooth and rough strains of type III (Z), the "Karim Khan," and a Sonnei strain of *Shigella* were grown successfully. The virulent strain of type III (Z), when cultivated by the aeration method, showed a ratio of toxicity to protective power in mice that was not significantly different from that shown by this strain when grown by usual broth methods in small amounts (table 2).

TABLE 1

Rapid growth run, no. 11, Flexner III (Z) dysentery bacilli

SOURCE OF SAMPLE G SIGNIFIES GROWTH RUN	LIVE COUNT			MASS			TURBIDITY		
	1	2	3	4	5	6	7	8	9
	100M/ml	*Units[*]*	*1[+] log of values in column 2[+]*	*g/L*	*Units[*]*	*1[+] log of values in column 5[+]*	*Units[†]*	*Units[*]*	*1 + log of values in column 8*
Prerun inoculum[‡]	16						361		
G at −55/60 hr	1.6	0.33	0.52				36	0.33	0.52
G at 0 hr	4.8[§]	1.00	1.00	1.77	1.00	1.00	119	1.00	1.00
G at 2 5/60 hr	44	9.17	1.96	2.39	1.35	1.13	1,098	9.23	1.96
G at 3 5/60 hr	102	21.2	2.33	3.50	1.98	1.30	2,499	21.0	2.32
G at 4 5/60 hr	129	26.9	2.43	4.22	2.38	1.38	3,010	25.3	2.40
G at 5 5/60 hr				4.68	2.64	1.42	3,540	29.7	2.47
G at 6 5/60 hr							3,720	31.2	2.49

[+] Log values are increased by one to avoid negative values.

[*] Based on value of unity arbitrarily assigned at zero time.

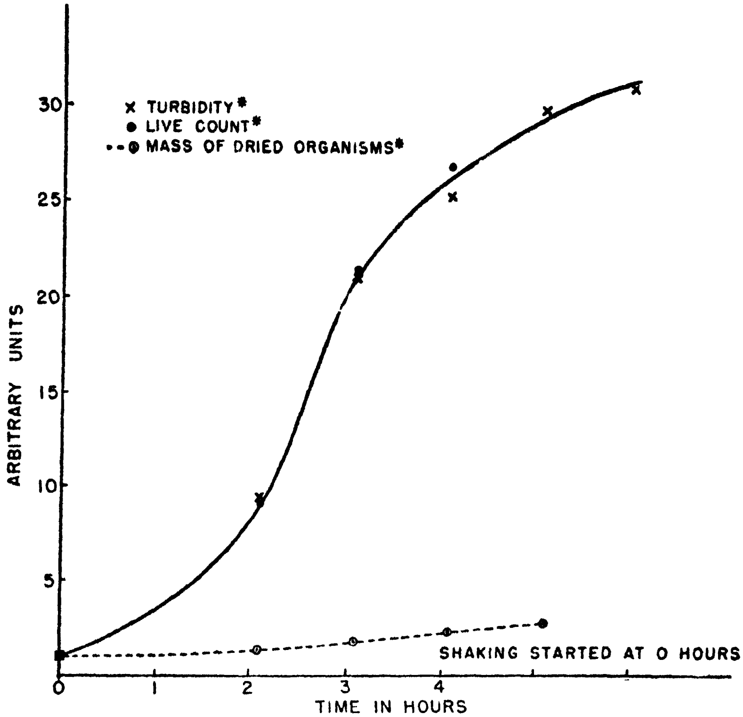
[†] Arbitrary units of concentration expressed as 1,000 (2 − log T), where T = % transmission; 800 units represent 1 gram of dried organisms at higher concentration levels.

[‡] This 17-hr-old culture was grown by ordinary methods but in the enriched medium described; 100 ml of this culture were used as an inoculum for each liter of medium in the shaking flasks. The two values recorded are about one-tenth of those reached by the growth curves at the end of the shaking period (see also figure 1).

[§] This one value was taken from a graph constructed from the other, experimental, live count data in this column.

The apparatus may be set up also to operate continually day and night with fresh medium constantly flowing into the flasks and fully grown harvest being drawn off at the same rate. Under the continual ingress of medium and egress of harvest, the type Z organisms grew at a very rapid rate. At the end of 96 hours of continuous growth, the bacteria were multiplying as well as during the early phases of the period. Because of difficulties in maintaining a steady flow of fresh medium and harvest, the average yield was about 3 g of bacteria (dry weight) per liter every 3 hours. Thus for the continuous operation there was a 40- to 50-fold increase over that rate of growth which takes place under ordinary methods of culturing.

This extremely rapid rate of growth is a very sensitive indicator of culture conditions. For example, restriction of growth to a concentration one-tenth that of the maximum occurred in one flask when the diameter of the air inlet tube was decreased to one-fourth of that leading to the other flask. Also it was found that whereas ordinary growth was easily achieved with the virulent type Z organisms, rapid regeneration was not accomplished until potassium salts were added to the medium. It seems likely that the effects of various substances and conditions on growth rate could be measured sensitively by the estimation of both rate of growth and maximal concentration under the conditions of this method.



*VALUES ARE EXPRESSED AS RELATIVE INCREASES OVER ARBITRARILY ASSIGNED UNITY AT ZERO TIME, TABLE I, COLUMNS 2,5 AND 8

FIG. 1. SIMULTANEOUS CHANGES IN MASS, TURBIDITY, AND LIVE COUNT DURING GROWTH OF FLEXNER III (Z) DYSENTERY BACILLI

Two explanations have been advanced for the slowing and final cessation of growth at the end of the logarithmic phase. One, the older, ascribes the interruption of growth to a gradual accumulation of inhibitory substances derived from bacterial metabolism during the growth of the culture. The other is supported by evidence indicating that the limits of necessary conditions for growth are exceeded by the demands of the large number of organisms present at the end of the logarithmic growth phase (Topley and Wilson, 1936). If the enriched medium, used in the method described here, was not shaken but was merely incubated for 18 to 20 hours, the concentration of organisms was about one-tenth that achieved in the ensuing 2½ hours of shaking (table 1). As there was essentially only one variable, it is apparent that shaking was responsible for the continuation of growth as well as for the increased growth rate. Elucidation of the mechanism of this effect of shaking should contribute, therefore, to the understanding of the factors ordinarily responsible for cessation of growth.

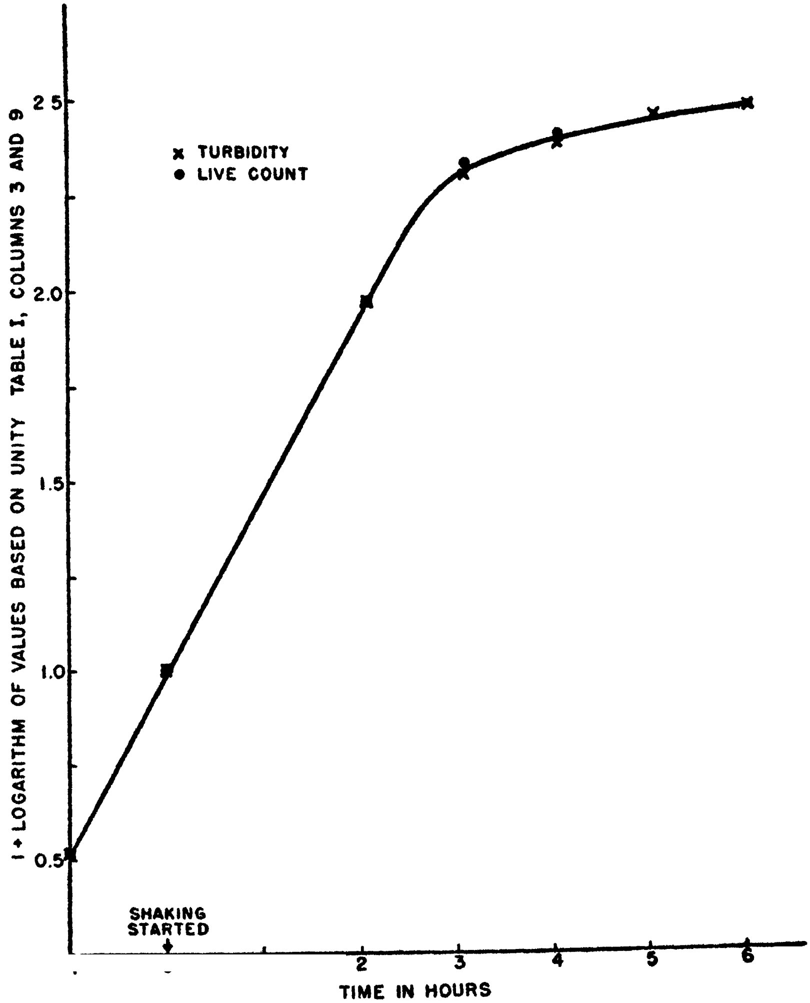


FIG. 2. LOGARITHMIC CHANGES IN LIVE COUNT AND TURBIDITY DURING GROWTH OF FLEXNER III (Z) DYSENTERY BACILLI

A number of possible explanations of the shaking effect are suggested. One is that through the increased surface area, greater accessibility to the oxygen supply is provided. Another is that shaking permits volatile toxins, such as excess of carbon dioxide, to be removed more rapidly, or others to be oxidized.

Another possibility is that the production of inhibitory substances proceeds more slowly in the presence of adequate supplies of oxygen. Various combinations of these effects also provide possible explanations for the observations.

Among the numerous unsolved problems concerning the nature of growth in normal and pathological cells is the fact that little is known concerning the potentialities for growth which are inherent in a system of normal cells when the only variables are the accessibility and rates of removal of metabolites. The rates of growth achieved here clearly show how tremendous these potentialities are. They suggest the possibility that rate of growth as well as its extent in biological systems can be altered greatly by merely changing the rate of supply and perhaps removal of metabolically active substances. It may not be necessary to assume that there is any change in cancer cells other than that producing a slight change in the rate at which nutrient substances and oxygen are made available to the cellular mechanism. For example, this might involve only a change in permeability of the cell membrane.

TABLE 2

Comparison of different methods of culture of Flexner III (Z) dysentery bacilli

A: Ordinary broth-grown organisms, centrifuged and washed with saline, killed by heat at 60 C for 30 minutes

B: Organisms from continuous, rapid-growth run, treated in same way as A

TOXICITY	PROTECTION	TOXIC UNITS*
(Dilution equal to 1 toxic unit)	(Dilution equal to 50% deaths)	(Required to give 50% protection)
A—1:97 B—1:52	A—1:19.7 B—1:13.9	A—4.9 B—3.7

* The vaccines were compared by toxicity-protection test in mice (Barnes and Dewey). The data were analyzed by Mrs. Estelle Brown with the Reed-Muensch method.

Rates of access and removal of metabolites with respect to bacterial cells may be a factor in the bacterial growth rate within the body of a host, and hence of importance in the virulence of invading organisms. The adaptation of bacteria to new environments may also be influenced by this same condition. Finally, this rapid adaptation of growth mechanisms which are ordinarily highly patterned is compatible with the findings of Schoenheimer (1942) and his school, namely, that synthetic and degradative cellular processes are going on in fundamental cellular components rapidly and continuously.

EXPERIMENTAL PROCEDURES

Procedure. One hundred ml of an 18- to 20-hour-old culture of *Shigella* organisms are added to each of two 6-liter Florence flasks containing a liter of culture medium apiece. The flasks are shaken mechanically at 37 C while sterile air is passed into the air space above the liquid. The rate of growth may be estimated by withdrawing, at intervals, 1 to 10 ml of medium through outlets near the bottom of the flasks and, after suitable dilution, measuring the turbidity by transmitted light in an electric photometer. A filter is used which transmits a band of light having a mean wave length of 660 mμ. By this means one can follow the growth curve of the organisms. By plotting the unit concentration of organisms, 1,000 (2-logarithm of the transmission), against the passage of time, one can relate any later readings to the curve so obtained (figure 3), thus permitting removal of the culture at any phase of growth. If desired, a unit of concentration on the graph may be easily standardized in terms of milligrams of nitrogen or grams of dried organisms per liter.

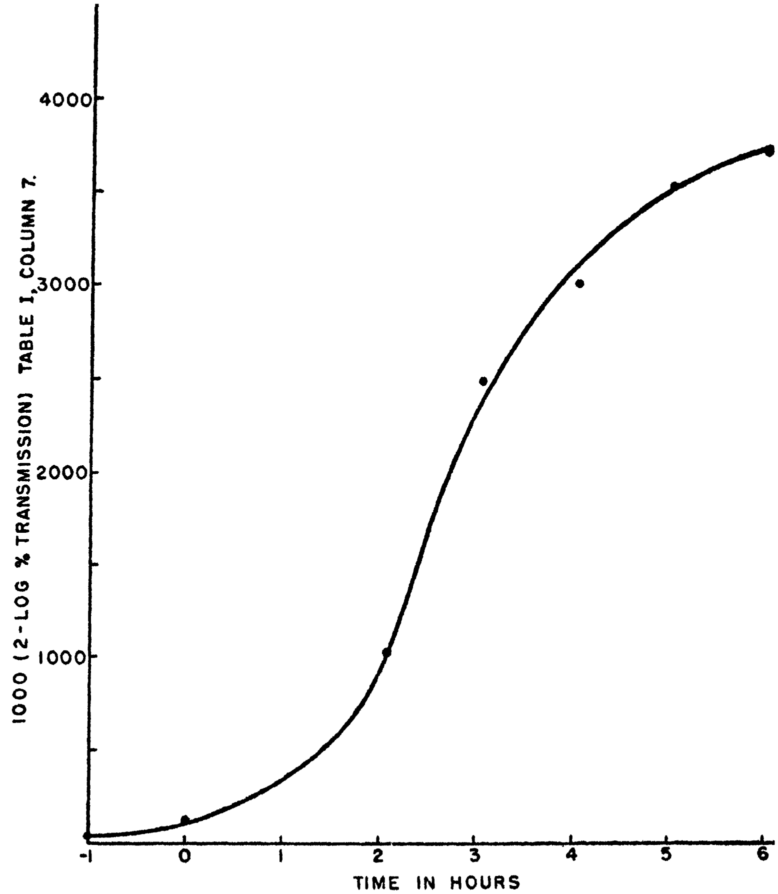


FIG. 3. GROWTH CURVE OF FLEXNER III (Z) DYSENTERY ORGANISMS BASED ON MEASUREMENTS OF TURBIDITY

Near the end of the logarithmic phase of growth, which occurs about 4 to 4½ hours after the shaking of the first batch has begun, the harvest is tapped off and a liter of fresh medium is added to each flask, a suitable inoculum always being present in the foam and draining medium left in the flasks from the previous run. The organisms will now grow to the previous maximum concentration in 2½ hours, having remained in the logarithmic phase of growth.

After the growth curve of the organism in this apparatus has been characterized adequately, it is possible to operate on a continuous 24-hour a day basis. For the first liter in each flask the procedure is the same as described above. When the growth of organisms in this first liter approaches the end of the logarithmic phase of growth, fresh medium is started flowing continuously into the two shaking flasks, and at the same rate the fully grown culture material is allowed to flow out of the flasks at the bottom. This is collected in large bottles, to which a bactericidal agent is added from time to time.

Media. The medium used is much more concentrated than the usual medium and is composed of the following constituents per liter:

Nutri peptone (Wilson)	20 g
Bacto beef extract (Difco)	10 g
Na_2HPO_4 $12H_2O$	18 g
KH_2PO_4	2.7 g
NaCl	1.0 g
Glucose	5.0 g
Nicotinic acid	0.0001 g

All of these constituents except the nicotinic acid and sugar are dissolved in the required amount of water, heated to 60 C, filtered hot through coarse filter paper, and then filtered in 2-liter lots through sterile Seitz filters. This medium remains clear. For large amounts, as the Seitz filtration is awkward, after filtration through paper the medium is autoclaved. Following this a moderate amount of precipitate settles out on standing; this can be removed by siphoning off the overlying medium. The nicotinic acid is dissolved in water (10 mg in 100 ml), sterilized 10 minutes in the autoclave, and added to the medium at the time of addition of the organisms. The glucose also is dissolved separately in some of the total water as a 5 per cent solution and sterilized separately.

Apparatus. The photograph (figure 4) shows the apparatus set up for continuous automatic use. However, if one wishes to grow only a few liters at a time, a large flask is not needed above the apparatus as a source of medium. The inlet tube for the medium (detail of area 6) in each shaking flask is then fitted up with a rubber stopper, and the outlets (12) are left plugged with cotton and unattached to rubber tubing (18 and 19). The medium is then introduced, a liter at a time, by siphons from small flasks.

The two central flasks (A and B) are the essential features. They are 6-liter Florence flasks with a small outlet tube fused into the side of each near the bottom. In the outlet tube is a glass stopcock (12). The stoppers in the flask necks are rubber, with three holes (see detail of area 6): one for air through which passes 7-mm glass tubing, one for introduction of the medium with 12.5-mm tubing, and one for an air vent (with 7-mm glass tubing) for which one or two ordinary distilling traps will suffice. The stoppers with these three glass fittings are wired firmly into the necks of the flasks, then covered with cotton and paper. Air is obtained directly from the compressed air line in the laboratory, is passed through 100 ml of concentrated sulfuric acid (22), then through an air trap (23), and finally through a sterilized drying tube (9) filled with cotton.

There must be no restrictions of any kind in the two air lines after the air leaves the T-tube (7).

The two Florence flasks are placed on a platform (13) and held in place by shallow metal receptacles (made from tin cans and attached to the platform) and

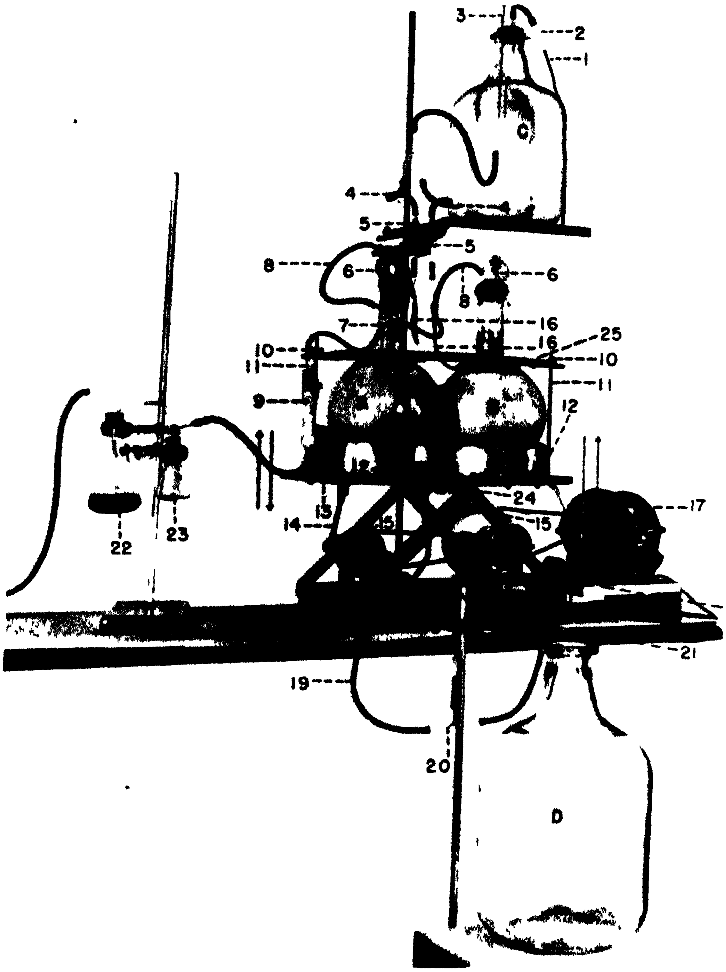


FIG. 4

by a plywood yoke (25) through which the necks of the flasks protrude. The yoke is held down at each end by a long bolt (11) and a wing nut (10). The flasks are well padded. The platform is made to move up and down on each side of an axis (24) in the direction of the arrows by an electric motor (17). A crank shaft is operated from the motor pulley (15) at 170 rpm, the platform and crank being joined to a wrist pin on the lower part of the platform by a pitman (14) from a crank pin. There is an excursion of the platform of 4 cm opposite the center of each flask. The center of the flasks is opposite a point

12 cm from the fulcrum (24) of the platform. The motion is just enough to keep the contents constantly and vigorously tossed about without causing them to strike violently against the walls.

For continuous operation the apparatus is set up as pictured so that media may be led in constantly from the upper bottle (C) through the siphon (3). The siphon may be started by blowing through a tube (1) and cotton filter (2). The rate of inflow is measured by means of drip counters (5) and controlled by clamps (4). The medium is run into each flask from the counters (5) through rubber tubes (16) at about one liter every two hours. The outlet tube (21) is raised or lowered to change the rate of egress of fluid. To permit better visualization, the outlet tube is pictured too low in the photograph and is usually placed about one-half inch below the level of fluid in the flasks when the platform is in a horizontal position. At this point the outlet behaves quite automatically and will control the level of liquid in the flasks within fairly wide limits of inflow of medium from above. One may control the operation through estimation of turbidity of the combined outflow from time to time. At times the turbidity of the medium from each flask may need to be measured separately.

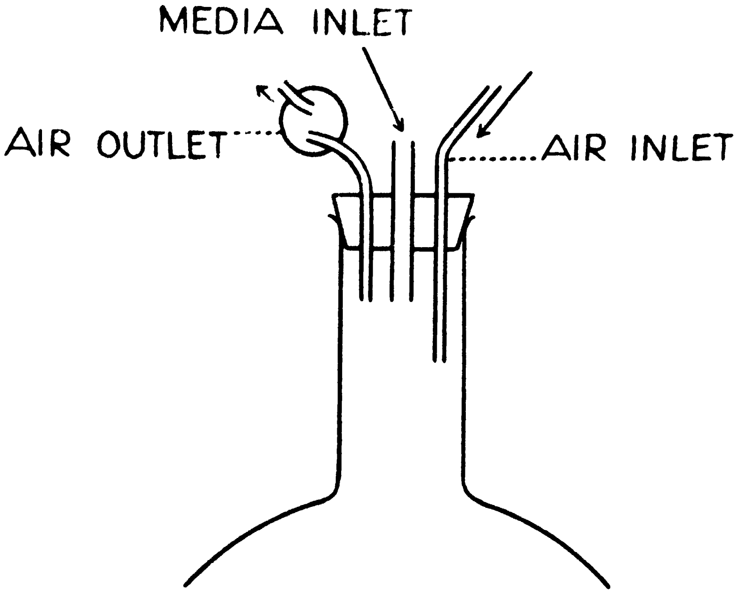


FIG. 5. DETAIL OF AREA AT NO. 6, FIGURE 4

ACKNOWLEDGMENT

We wish to thank Dr. Merlin Cooper for suggestions and Mr. J. Bell for constructing the shaking apparatus.

SUMMARY

A method is described by which *Shigella paradysenteriae* organisms may be grown continuously at the rate of 4 g (dry weight) per liter per 2½ hours with simple laboratory apparatus. Certain relationships of the findings to problems of growth are noted.

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TWO ANTIBIOTICS PRODUCED BY A STREPTOMYCES

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The genus *Streptomyces* has gained considerable recognition in the field of antibiotics. Outstanding members of the genus to date are *Streptomyces antibioticus* (Waksman and Woodruff, 1941), *Streptomyces lavendulae* (Waksman and Woodruff, 1942), and *Streptomyces griseus* (Shatz *et al.*, 1944), which elaborate actinomycin, streptothricin, and streptomycin, respectively. The streptomyces isolate studied in this work bears a resemblance to *S. lavendulae* but has morphological and biochemical characteristics that differentiate it from this described species. Moreover, by biological and chemical tests, the antibiotic activity of the isolate has been found to result from a mixture of at least two antibiotic substances. Subsequent chemical fractionation has yielded one material in relatively pure form. However, the second active fraction has not been fully separated from the first. A more complete study of the purified fractions will be reported later.

This paper covers the production of antibiotic activity, the preparation of crude concentrates, activity tests, and preliminary attempts to separate the active components. Some of the methods and techniques applied to determine the dual nature of the antibiotic activity may be of service to others confronted with an apparently new biologically active substance.

EXPERIMENTAL RESULTS

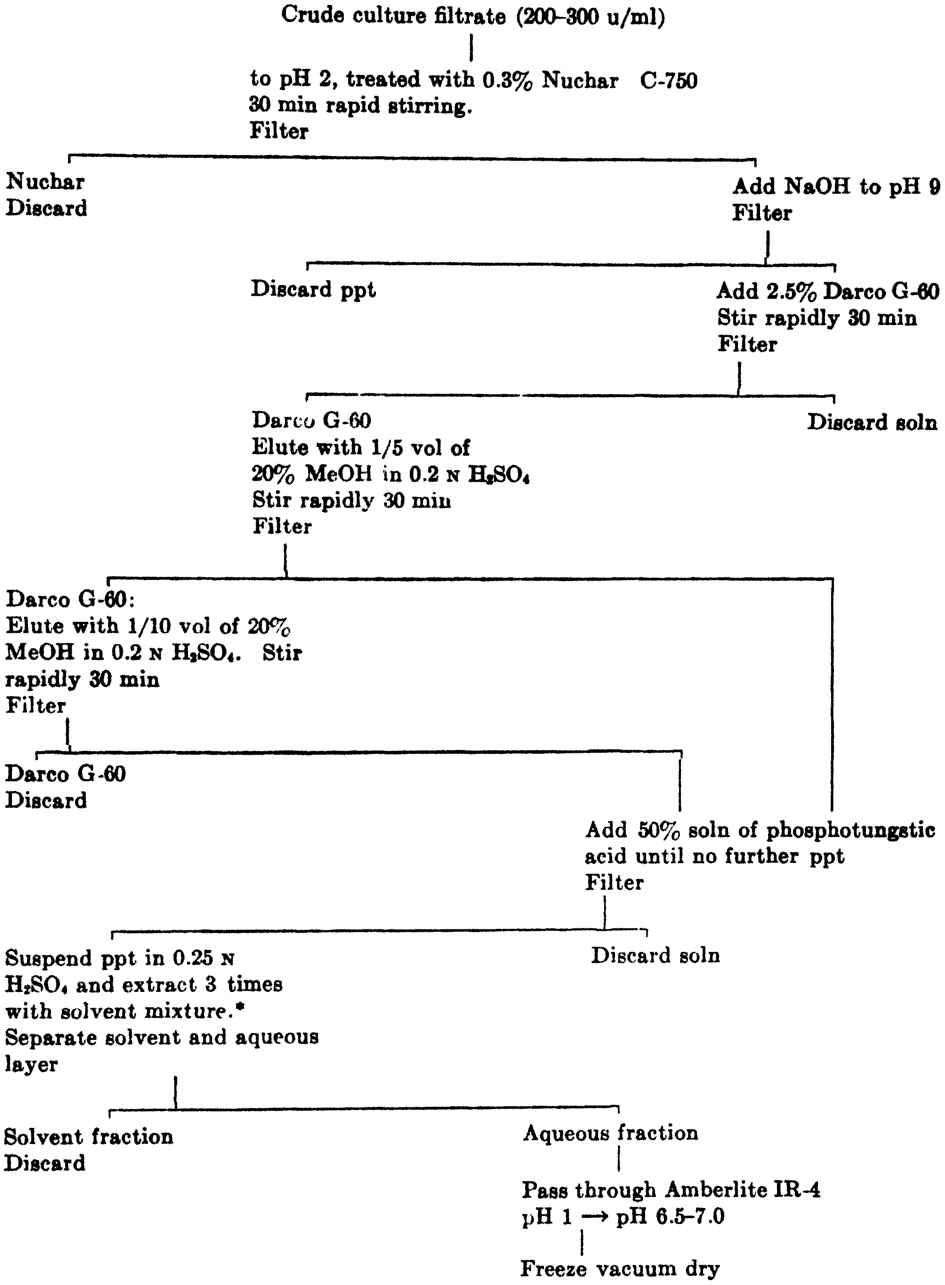
Isolation, description, and identification of the streptomyces isolate. The streptomyces was isolated by one of us (C. F.) from a contaminant on a petri plate seeded with *Brucella abortus*. The presence of the contaminant was characterized by a large zone of inhibition surrounding the streptomyces colony. This contaminant was picked and subjected to extensive study for the production of the antibiotic material. Extreme care was exercised at the outset to ensure a pure culture, but to ensure further its purity, single colonies were picked from agar plate dilutions of spores of the mother culture and propagated individually to afford sufficient inoculum for shake cultures. Following growth of the cultures in shake culture flasks for 5 days, the broths from 20 such spore isolates were assayed for potency by the *Bacillus subtilis* cup assay method and also subjected to bacterial spectrum analysis by the agar streak method with streptomycin-resistant test organisms (methods described below). Although the yield of active material varied appreciably among the 20 cultures, their bacterial spectra were similar. It was concluded that the streptomyces culture was pure insofar as the antibiotic activity of individual spore cultures was concerned.

Together with *S. lavendulae* (Waksman no. 10 strain) and *S. griseus* (Waksman no. 10 strain), the newly isolated streptomyces was examined systematically for its morphological and biochemical characteristics according to the procedures of Waksman (1919). To ensure stabilized cultures, the three organisms were first maintained on sterile soil for 5 weeks. The *S. lavendulae* and *S. griseus* cultures were found to coincide closely with the descriptions presented by Waksman. However, the newly isolated culture did not sufficiently resemble any species in Waksman's key to justify ascribing a known species name to it.

On the diagnostic media the isolate displayed distinct differences from *S. lavendulae* in type and amount of growth as well as in soluble pigment production. On Dorset's egg medium *S. griseus* produced a wrinkled, yellow growth, formed no soluble pigment, and caused no liquefaction; *S. lavendulae* produced a wrinkled purple growth, formed a pink soluble pigment, and caused liquefaction; whereas the streptomyces isolate produced a clay-colored growth, formed a brownish-drab soluble pigment, and caused no liquefaction. On Loeffler's blood serum *S. griseus* appeared as a yellow growth, produced no soluble pigment, and caused liquefaction; *S. lavendulae* appeared as a light cinnamon-colored growth, produced no soluble pigment, and caused no liquefaction; the isolate appeared as a clay-colored growth, produced a pink soluble pigment, and showed questionable liquefaction. Soluble pigments were produced on glycerol nitrate by *S. griseus* (yellow) and by *S. lavendulae* (brown), but not by the isolate. On this medium the aerial hyphae of *S. griseus* were water green, of *S. lavendulae* lavender, and of the isolate pallid mouse gray. Moreover, sharp differences were observed in the structure of aerial hyphae. *S. griseus* produced straight sporogenous hyphae, *S. lavendulae* slightly coiled hyphae, and the isolate tightly coiled hyphae. The most striking biochemical difference was the failure of the isolate to liquefy gelatin or peptonize milk after 1 month of incubation at 25 C.

Assay and production of the antibiotic material. Solutions of the antibiotic were assayed by the "penicylinder" plate method, with *B. subtilis* Marburg as the test organism. The diameters of the zones of inhibition, when plotted against the logarithm of the dosage, exhibited a linear relation, and the slope of the curves was similar to that obtained with streptomycin. This relation was found also when the test organism was *B. subtilis* ATCC 6633, *B. mycoides* (Waksman), and *Serratia marcescens* (Waksman). Accordingly, in evaluating the activity of solutions of the new antibiotic, all samples were assayed against a streptomycin standard, and the values were expressed as equivalent to streptomycin units assayed against *B. subtilis*. For routine assays, large plates of the type described by Beadle *et al.* (1945) were employed. The streptothricin unit also was standardized to the foregoing streptomycin unit.

Propagation of the streptomyces for production of the antibiotic was almost exclusively by submerged culture in shake culture flasks (300 to 400 ml of liquid) or in aerated fermenters (10 liters and 200 liters of liquid). When a medium containing soy flour hydrolyzate, glucose, and salts or a medium containing soy flour, corn steep, glucose, and salts was used, yields of 200 to 300 units per ml were secured consistently in 4 to 5 days. Variation of incubation temperatures between 24 and 29 C did not noticeably change the rate of formation or the



* Solvent mixture: 120 parts diethyl ether, 100 parts *n*-amyl alcohol, and 5 parts ethyl alcohol.

maximum yield of the antibiotic material. Incubation at 37 C slightly depressed the final yield.

Purification of antibiotic material. Spot tests on clarified culture filtrate showed the active materials to be heat-labile (boiling or autoclaving at 15 pounds' steam pressure for 30 minutes), acid-stable (pH 1 for 2 hours), alkali-labile (pH 11.5 for 2 hours), adsorbable on activated carbon, precipitable by phosphotungstic but not by trichloracetic acid, and not extractable at pH 3.0 or 8.1 by ether, chloroform, amyl acetate, or ethyl acetate.

On the basis of the foregoing and subsequent data, the accompanying method of processing was evolved.

The foregoing purification procedure, when culture filtrate of 200 to 300 u per ml is employed, has given a recovery of 40 to 55 per cent of the activity, as 400 to 600 u per mg material. A 190-liter batch gave a recovery of 39 per cent at 592 u per mg; the only other large run to date, 230 liters, gave a recovery of 53 per cent at 483 u per mg.

For convenience the antibiotic (when considered as a mixture of active fractions) will be designated in the remainder of the paper as "F."

Comparison of antibiotic "F" with streptothricin and streptomycin. Bacterial spectrum analysis of the three antibiotics by the agar streak method (4 per cent tryptose agar, Difco) showed that the inhibitory action of antibiotic "F" resembled that of streptomycin, but "F" resembled streptothricin in its effect on the Bodenheimer organism (table 1).

A comparison was therefore made with this organism on a less nutritious medium, nutrient agar (as recommended for the streptomycin test by the Food and Drug Administration, July, 1946). The Bodenheimer organism was found to withstand more than 1,000 u per ml of streptomycin, whereas it was completely inhibited by 1 u per ml of antibiotic "F." A number of preparations of antibiotic "F" were tested in this way, and the effect was found to be consistent.

The three antibiotics were compared by the cysteine inactivation, iodine regeneration procedure of Denkelwater *et al.* (1945). According to these workers, streptomycin may be completely inactivated with cysteine and subsequently reactivated by treatment with iodine, but the activity of streptothricin, on the other hand, is not affected by cysteine. Table 2 shows the results of representative tests with cysteine and iodine. Some samples of antibiotic "F" responded to iodine regeneration, whereas others remained essentially unchanged. Tests on a large number of samples of the three antibiotics showed, on the average, that streptomycin samples were inactivated by cysteine with recovery to 90 per cent of the original activity on iodine treatment; streptothricin samples dropped to 75 per cent with cysteine and recovered to 95 per cent with iodine; and samples of antibiotic "F" dropped to 50 per cent with cysteine and recovered to 80 per cent with iodine. However, there was wide discrepancy among samples of antibiotic "F," particularly in regard to the percentage regeneration with iodine.

To determine whether bacteria made resistant to streptomycin would also be resistant to the new antibiotic, and vice versa, *B. subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were carried through eight transfers on nutrient agar (FDA streak plate agar) containing increasing concentrations of one antibiotic. The resulting resistant cultures were tested against the other antibiotic. All the organisms developed resistance more readily against streptomycin than against "F." The data are summarized in table 3. *B. subtilis*, *E. coli*, and *P. acruginosa* were modified to withstand 1,000 u per ml of streptomycin, the highest concentration of antibiotic used. Only *P. aeruginosa* became resistant to 1,000 u per ml of "F"; the remaining three species withstood not more than 1 u per ml after eight transfers.

TABLE 1

Bacterial spectra of streptomycin, streptothricin, and antibiotic "F" (tryptose agar medium)

Numbers represent amount of growth compared with control cultures: 4 = equivalent to control; 0 = no growth. Incubation at 37 C for 48 hours

ORGANISM	STREPTOMYCIN U/ML					STREPTOTHRICIN U/ML					ANTIBIOTIC "F" U/ML				
	0.3	1	3	10	30	0.3	1	3	10	30	0.3	1	3	10	30
B. subtilis Waksman	3	2	1	0	0	4	3	3	0	0	3	1	1	0	0
E. coli Merck	3	2	2	1	0	4	4	3	2	1	3	2	2	1	0
M. phlei ATCC 355	4	1	0	0	0	4	4	4	1	0	4	1	0	0	0
B. megatherium	4	4	2	0	0	4	4	4	4	2	3	3	1	0	0
S. aureus 209	4	4	2	1	0	4	4	4	3	2	4	3	2	1	0
Brucella abortus 7705	4	1	0	0	0	4	4	3	0	0	4	1	0	0	0
Brucella abortus 19	4	1	0	0	0	4	4	3	1	0	4	1	0	0	0
Brucella melitensis	4	1	0	0	0	4	4	4	1	0	4	2	0	0	0
Brucella suis	4	1	0	0	0	4	4	4	1	0	3	1	0	0	0
B. subtilis 6633	3	0	0	0	0	4	3	1	0	0	2	0	0	0	0
E. coli Waksman	3	2	2	1	0	4	4	3	2	1	3	2	1	1	0
B. mesentericus	3	3	2	1	0	4	4	4	4	4	3	3	1	1	0
S. aureus 24T	4	3	3	2	1	4	4	4	3	2	4	3	2	2	1
C. diphtheriae	3	1	0	0	0	4	4	4	4	1	4	1	0	0	0
K. pneumoniae	4	4	1	0	0	4	4	4	4	4	3	3	1	0	0
P. aeruginosa	4	4	4	3	2	4	4	4	3	1	4	4	4	4	2
S. enteriditis	3	2	2	1	0	4	3	3	2	1	2	2	1	1	0
B. mycoides Waksman	3	3	2	1	0	4	4	4	4	4	4	3	2	1	0
S. hemolyticus	4	4	2	0	0	4	4	4	4	1	4	3	2	0	0
B. subtilis Marburg	4	4	4	3	2	4	4	4	3	3	3	3	2	2	1
S. albus	4	4	1	0	0	4	4	4	4	0	4	4	1	0	0
S. marcescens Waksman	4	3	2	1	0	4	4	4	3	2	3	3	2	1	1
E. typhi	4	4	3	2	1	4	4	3	2	1	4	3	1	0	0
S. schottmuelleri	4	4	3	2	1	4	4	3	2	1	4	3	2	1	0
S. suipestifer	4	4	3	2	1	4	4	3	3	1	4	2	2	0	0
*Bodenheimer**	4	4	4	4	4	4	4	4	1	1	4	4	3	1	0

* This organism was isolated by Dr. Bodenheimer of the College of Physicians and Surgeons, Columbia University, New York City.

When the foregoing experiment was repeated with a different preparation of "F" (organisms carried through 14 transfers in the presence of the antibiotics), it was observed that not only were "F"-resistant organisms generally as resistant to streptomycin, but streptomycin-resistant organisms were (with the exception of *B. subtilis*) equally resistant to antibiotic "F" (table 3).

TABLE 2

Cysteine inactivation, iodine regeneration of antibiotics

SAMPLE	UNTREATED u/ML*	AFTER CYSTINE		AFTER I_2 REGENERATION	
		u/ml	% of original	u/ml	% of original
F-3	484	315	65	389	80
Streptomycin	550	42	8	513	93
F1-2A-10	550	306	56	516	94
F3-1	506	354	70	504	100
Streptomycin	597	30	5	582	98
Streptothricin	220	178	81	252	114
F31-35-7	400	111	28	295	74
F1-2A-10A	705	362	51	414	59
Streptomycin	160	tr		134	84
F31-35-7	400	82	20	308	77
F4-7-3C-10-2	215	96	45	212	98
F1-2A-10A	705	447	64	447	64
Streptomycin	160	tr		144	90
Streptomycin	427	tr		367	86
Streptothricin	220	165	75	216	98

* For ease of comparison, all original solutions are based on 1 mg per ml, so that figures in this column also indicate u per mg (i.e., degree of purity).

TABLE 3

Comparison of cultures made resistant to streptomycin (S) and antibiotic "F" by serial transfer

ORGANISM	TREATMENT	MAXIMUM CONC. PERMITTING GROWTH, u/ML			
		8 transfers (EXPT. 1)		14 transfers (EXPT. 2)	
		S	"F"	S	"F"
B. subtilis 6633	Unmodified	0.1	0.1	1.0	1.0
	S-resistant	1,000	0.1	1,000	30
	F-resistant	10	0.3	30	30
E. coli Waksman	Unmodified	0.1	0.1	3	3
	S-resistant	1,000	0.3	1,000	1,000
	F-resistant	1.0	1.0	10	10
S. aureus 209	Unmodified	0.1	0.1	3	1
	S-resistant	1.0	0.3	30	30
	F-resistant	1.0	1.0	30	10
P. aeruginosa	Unmodified	3	3	10	10
	S-resistant	1,000	300	1,000	1,000
	F-resistant	1,000	1,000	1,000	1,000

The preceding results gave the first definite indication that preparations of "F" varied as to their antibiotic constitution. Accordingly, for a group of "F" preparations obtained by slightly different procedures, activities were determined by the afore-mentioned plate assay with *B. subtilis* and *E. coli* against a streptomycin standard. The *E. coli* and *B. subtilis* values and their ratios are given in table 4. Whereas most ratios approximated 1.00, five preparations gave low ratios.

Fractionation of antibiotic activity of "F." As has been shown, preparations of "F" resemble streptomycin in their bacterial spectra (notable exception, Bodenheimer organism). This similarity was borne out in chemical and physical tests. Both antibiotics gave a positive Sakaguchi reaction, yielded maltol (Schenck and Spielman, 1945) on alkaline hydrolysis, gave positive oxidized nitroprusside tests, showed only end absorption in the ultraviolet range of the spectrophotometer, and could be processed from culture filtrates in the same manner. However, lack of correspondence of the "F" preparations to streptomycin was indicated by the cysteine inactivation, iodine regeneration experiments, *E. coli*:*B.subtilis* ratios, and by the susceptibility of streptomycin-resistant cultures to some preparations of "F." These data suggested that antibiotic "F" was a mixture of biologically active substances, one of which might be streptomycin or streptomycinlike. The variation in similarities and dissimilarities of streptomycin and different "F" preparations seemed to indicate variation in the biologically active components of "F."

TABLE 4

E. coli:*B. subtilis ratios of "F" preparations*

NO.	E. COLI, U/MG	B. SUBTILIS, U/MG	E. COLI:B. SUBTILIS RATIO
1	684	579	1.18
2	512	450	1.14
3	453	424	1.07
4	406	428	0.95
5	438	456	0.96
6	250, 203	500	0.50, 0.41
7	482	458	1.05
8	380	380	1.00
9	470	540	0.87
10	340, 460	716	0.48, 0.64
11	488	470	1.04
12	528	469	1.12
13	416	388	1.07
14	304	369	0.82
15	249	290	0.86
16	279, 284	555	0.50, 0.51
17	126, 128	234	0.54, 0.55
18	418, 371	583	0.72, 0.64
19	851	756	1.12
20	816	821	0.99
21	715	766	0.93
22	503	635	0.79
23	327	377	0.86

In view of the foregoing considerations it was decided to investigate "F" preparations for mixtures of antibiotics. First, streptomycin-resistant "F"-susceptible, and streptomycin-susceptible "F"-resistant, bacteria were sought from a natural source (soil) to provide means for differentiation. About 20 bacteria were readily isolated that were resistant to 1,000 u per ml of streptomycin and susceptible to 1 to 100 u per ml of "F" by the agar plate streak method. Attempts to find bacteria resistant to appreciable quantities of "F" were unsuccessful.

By the use of these recently isolated test bacteria and also the Bodenheimer organism, spectrum analyses were conducted on preparations of "F" and on fractions that were obtained by chromatographic treatment (Brockman alumina column). The data are given in table 5. Streptomycin and streptothricin were included for comparison. Each antibiotic was used at levels of 0.3, 1.0, 3, 10, 30, 100, 300, and 1,000 u per ml.

TABLE 5

Bacterial spectra of "F" preparations

Figures represent u per ml (*B. subtilis* assay) of antibiotic just preventing growth of test organism. Tryptose agar used. Incubation 24 hours at 28 C. Streak agar plate technique employed

ANTIBIOTIC	U/MG	TEST ORGANISM										
		No. 3	No. 4	No. 6	No. 10	No. 12	No. 14	No. 15	No. 17	No. 21	No. 28	Bodenheimer
Streptomycin	440	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	1,000	>1,000	>1,000	>1,000
Streptothricin	270	10	30	3	30	3	1	100	1	>100	30	30
F no. 1	705	30	30	3	30	10	3	30	10	100	30	100
F no. 2	617	10	30	10	30	10	10	30	3	100	100	30
F no. 3	579	10	30	3	10	3	1	30	3	300	10	30
F no. 4*	800	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	100	>1,000	>1,000
F no. 5*	250	1	3	1	1	1	1	3	1	100	3	10
F no. 6*	150	1	1	1	1	3	1	3	1	100	3	30

* Fractions from chromatographic column.

Table 5 indicates some spectrum differences among original preparations of "F" (nos. 1, 2, and 3) suggesting that different lots vary biologically. In general, however, their spectra correspond closely to that for streptothricin.

When an "F" preparation was fractionated chromatographically, antibiotic fractions of a type corresponding to no. 4 were obtained from the first elution. This material not only resembled streptomycin biologically, but also by such chemical tests as quantitative Sakaguchi determination, complete inactivation with ketone reagents, maltol formation on alkaline hydrolysis, specific rotation, and chemical analysis. Following elution of the streptomycinlike material, a second antibiotic material was obtained of a more streptothricinlike character (nos. 5 and 6).[1]

Since the foregoing work strongly indicated that "F" preparations were composed of at least two antibiotic substances, efforts were directed to evolving a procedure for assaying one antibiotic in a mixture. The isolated streptomycin-resistant "F"-susceptible bacteria were examined in an attempt to obtain a suitable test organism. Although some gave favorable results, the Bodenheimer organism was most suitable. With this organism there was a linear relation between the diameter of the zone of inhibition and the logarithm of the dosage between the limits of 5 and 40 u per ml of streptothricin.

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[1] Richardson, E. R., Trussell, P. C., and Grant, G. A., unpublished data.

Streptomycin alone did not inhibit the Bodenheimer organism at concentrations of 1,000 u per ml, but it enhanced the inhibitory effect of streptothricin when both were present. Gradational increases in inhibition of the test organism were observed when streptothricin concentration was kept constant while that of streptomycin was increased from 100 to 400 u per ml; but from 400 to 1,000 u per ml of streptomycin did not further increase the inhibition. Accordingly, all dilutions of the streptothricin standard as well as those of "F" were made to contain 400 u per ml of streptomycin. Good reproducibility was thus obtained in assays of "F" preparations for streptothricinlike activity, the results of which are given in table 6. This shows that different preparations may vary from <2 to 68 per cent streptothricinlike material. The no. 8 series consists of consecutive fractions from a chromatographic column. Appreciable amounts of the streptothricinlike material are present in the last three fractions, and relatively little in the first three.

TABLE 6

Percentage of streptothricinlike material in "F" preparations and fractions

PREP'N NO.	POTENCY, U/MG		
	B. subtilis	Bodenheimer	% streptothricinlike
1	438	60, 61, 74, 74	14, 14, 17, 17
2	569	10	<2
3	208	54	27
4	265	180, 175	68
5	136	35	26
6	583	332, 321	55
7	705	440	62
8D	989	<45	<4
8E	902	<15	<2
8F	778	<30	<3
8G	590	154	26
8H	570	200	35
8I	306	88	28

Rate of elaboration of antibiotic components of "F." Six shake flask cultures (150 ml) were assayed against *B. subtilis* and the Bodenheimer organism (cup assay) at 4, 5, and 9 days of incubation, to determine total antibiotic activity and streptothricinlike activity, respectively. The data appear in table 7. The Bodenheimer-active fraction was produced in much smaller amounts than the streptomycinlike fraction and reached its peak about the fifth day. The streptomycinlike fraction was produced chiefly after the fourth day, reaching a maximum at the ninth day. This difference in rate of formation is shown more clearly by the ratio of Bodenheimer organism activity to *B. subtilis* activity.

Intravenous toxicity. Preliminary tests were made on a number of column fractions: fraction I, streptomycinlike (not inhibiting Bodenheimer organism at 1,000 u per ml); and fractions II, III, and IV, containing 55, 30, and 65 per cent of streptothricinlike activity, respectively.

TABLE 7

Rate of formation of antibiotic components of "F" during fermentation

FERMENT NO.	4 DAYS			5 DAYS			9 DAYS		
	B. SUBT., u/ML	BODEN., u/ML	RATIO*	B. SUBT., u/ML	BODEN., u/ML	RATIO	B. SUBT., u/ML	BODEN., u/ML	RATIO
1	75	60	0.80	204	107	0.53	370	64	0.17
2	40	43	1.07	128	72	0.56	335	70	0.21
3	46	54	1.17	200	85	0.43	330	64	0.19
4	93	62	0.67	285	100	0.35	390	71	0.18
5	112	72	0.64	322	102	0.32	388	71	0.18
6	86	56	0.65	276	101	0.36	381	62	0.16
Average......			0.83			0.43			0.18

* Ratio, Bodenheimer organism activity: *B. subtilis* activity.

TABLE 8

Toxicity of "F" preparations

FRACTION NO.	NO. OF MICE	TOTAL UNITS INJECTED INTRAVENOUSLY	PURITY OF SAMPLE UNITS PER MG	STREPTOMYCINLIKE UNITS INJECTED	STREPTOTHRICINLIKE UNITS INJECTED	IMMEDIATE DEATHS	DELAYED DEATHS									
							Days									% Dead at end of 12 days
							1	2	3	4	5	6	7	8	9 to 12	
I	10	5,000	775	5,000	0	10										100
	10	3,000	775	3,000	0	4	0	0	0	0	0	0	0	0	0	40
	10	1,500	775	1,500	0	0	0	0	0	0	0	0	0	0	0	0
	10	750	775	750	0	0	0	0	0	0	0	0	0	0	0	0
II	10	1,000	530	450	550	0	0	0	5	4	0	0	0	0	0	90
	5	500	530	225	275	0	0	1	0	0	1	0	0	0	0	40
III	8	3,042	640	2,129	913	8										100
	8	1,521	640	1,065	456	0	0	0	1	0	0	0	0	0	0	12
IV	8	3,000	705	1,050	1,950	3	0	0	0	0	1	1	0	1	0	75
	8	1,500	705	525	975	0	0	0	0	1	0	0	0	0	0	12
	8	750	705	263	487	0	0	0	0	0	1	0	0	0	0	12

The results are summarized in table 8. The toxicity of fraction I is similar to that of streptomycin, the LD_{50} being 3,000 units. (This streptomycinlike fraction injected subcutaneously, 2,400 units daily, into each of 10 mice for 6 days produced no apparent ill effect after 10 days.) Fractions II, III, and IV, containing streptothricinlike material, showed delayed toxicity and were more toxic than fraction I.

More conclusive toxicity tests of the streptothricinlike activity await further separation of the antibiotic components.

In vivo activity. Protective properties of "F" compared to streptomycin are indicated in table 9.

Mice injected with a single intraperitoneal dose of 500 units of a preparation of "F" (65 per cent streptothricinlike) survived longer against a lethal dose of *Salmonella suipestifer* intraperitoneally than did mice receiving a similar dose of streptomycin. No significant protective action was noted when 1,000 units of "F" were given orally, whereas oral streptomycin had a slight effect. A single subcutaneous 500-unit dose of "F" extended more protection to mice against a lethal dose of *Salmonella schottmuelleri* intraperitoneally than did a similar dose of streptomycin.

TABLE 9

In vivo activity

NO. MICE	ANTIBIOTIC	SINGLE DOSE UNITS	ROUTE*	ORGANISM	% SURVIVAL		
					3 days	10 days	30 days
10	"F"	500	I.P.	*S. suipestifer*	90	0	0
10	Streptomycin	500	I.P.	*S. suipestifer*	33	0	0
10	None	0		*S. suipestifer*	0	0	0
10	"F"	1,000	Oral	*S. suipestifer*	0	0	0
10	Streptomycin	1,000	Oral	*S. suipestifer*	10	0	0
10	None	0		*S. suipestifer*	0	0	0
10	"F"	500	S.C.	*S. schottmuelleri*	100	90	70
10	Streptomycin	500	S.C.	*S. schottmuelleri*	100	60	20
10	None	0	S.C.	*S. schottmuelleri*	40	0	0

* I.P. = intraperitoneal injection; S.C. = subcutaneous injection.

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The authors are indebted to Mr. A. Barol, Wyeth Institute of Applied Biochemistry, for supplying the culture of the Bodenheimer organism, and to Dr. S. A. Waksman, New Jersey Agricultural Experiment Station, Rutgers University, for cultures of *S. lavendulae* and *S. griseus*; they also express their appreciation to Mr. Ivan Brown, Miss Joyce Wilson, and Miss June Perry for valuable technical assistance.

DISCUSSION AND SUMMARY

According to Waksman's classification key, the *Streptomyces* employed in this study is distinct from either *S. griseus* or *S. lavendulae*, as indicated from a determinative study of the three streptomycetes on 36 diagnostic media that included 7 carbon and 10 nitrogen sources. Purity of the isolate was assured by replating and by bacterial spectrum studies on culture filtrates from individual colonies of the mother culture.

Accordingly, the premise seems acceptable that the isolate is an organism not previously described and that it elaborates a mixture of antibiotics. Whether this mixture is composed of only two separate active fractions is as yet uncertain. Evidence favors the identity of one fraction with streptomycin. All that may be presumed of the second fraction at present is that it is like streptothricin.

Preparations containing the streptothricinlike component are toxic. The antibiotic mixture produced by this new isolate of *Streptomyces* has been found to possess *in vivo* activity.

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BACTERIOPHAGE UNDER THE ORDINARY MICROSCOPE[1]

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In reporting his experiences on the use of the ultramicroscope for examining bacteriophage, d'Herelle (1924) stated: "If one follows a bacillus culture inoculated with the ultramicrobe one sees that a certain number of the bacteria become deformed, first becoming swollen, then taking a spherical form, and at the same time very fine brilliant points appear in the interior of the bacilli. Then, suddenly, there is a rupture of the rounded bacillus and there remains in its place a slight cloud of protoplasm in which are embedded the brilliant particles. The protoplasm dissolves within a few minutes liberating the corpuscles into the medium." In his later book (1926), he repeated the statement that bacteriophage can be seen through the ordinary microscope.

In spite of the large amount of work done by d'Herelle, there has come to be a trend in this country toward the belief that bacteriophage is submicroscopic in size and enzymatic in nature (Bronfenbrenner, 1927). Usually, therefore, anyone who may see such particles is disinclined to believe that they are bacteriophage. Even one who believes that they are is inclined, in the face of the general skepticism, to be rather circumspect in the presentation of evidence of such a nature. This is probably the reason why MacNeal, Frisbee, and Krumwiede (1937) stated their observations in the following words: "The intracellular granules which retain the blue of the Castenada stain are abundant in the cholera vibrio altered by bacteriophage. The possibility that these granules represent the particles endowed with the property of reproduction and with the property of secreting the lytic enzymes of the bacteriophage principle is worthy of careful consideration."

In 1943 Luria, Delbruck, and Anderson reported that by the use of the electron microscope it was possible to see the particles of the bacteriophage for *Escherichia coli*. Baylor, Severens, and Clark (1944) reported similar results for the bacteriophage specific for *Salmonella pullorum*.

Source of biological material. In an effort to prepare bacteriophage for observation under the ordinary microscope, four strains of the pea nodule organism, *Rhizobium leguminosarum*, were used. The first, and most frequently studied, denoted B_4, is an isolation from a commercial legume culture used for inoculating peas, and was selected, from others similarly isolated, mainly because of its susceptibility to the action of bacteriophage. The other strains studied here were obtained from the University of Wisconsin, and are listed in that collection as nos. 303, 311, and 313.

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[1] Journal paper 704, New York State Agricultural Experiment Station, Geneva, N.Y., March 6, 1947.

Bacteriophage was obtained from three different soils in the neighborhood of Geneva, which were found, during an investigation of the subject, to contain relatively large numbers of bacteriophage particles specific for the pea nodule organism. All were obtained by incubating 1 ml of a 24-hr culture of B_4 in the medium of Campbell and Hofer (1943) for 24 hours and then filtering it through a Berkefeld N candle.

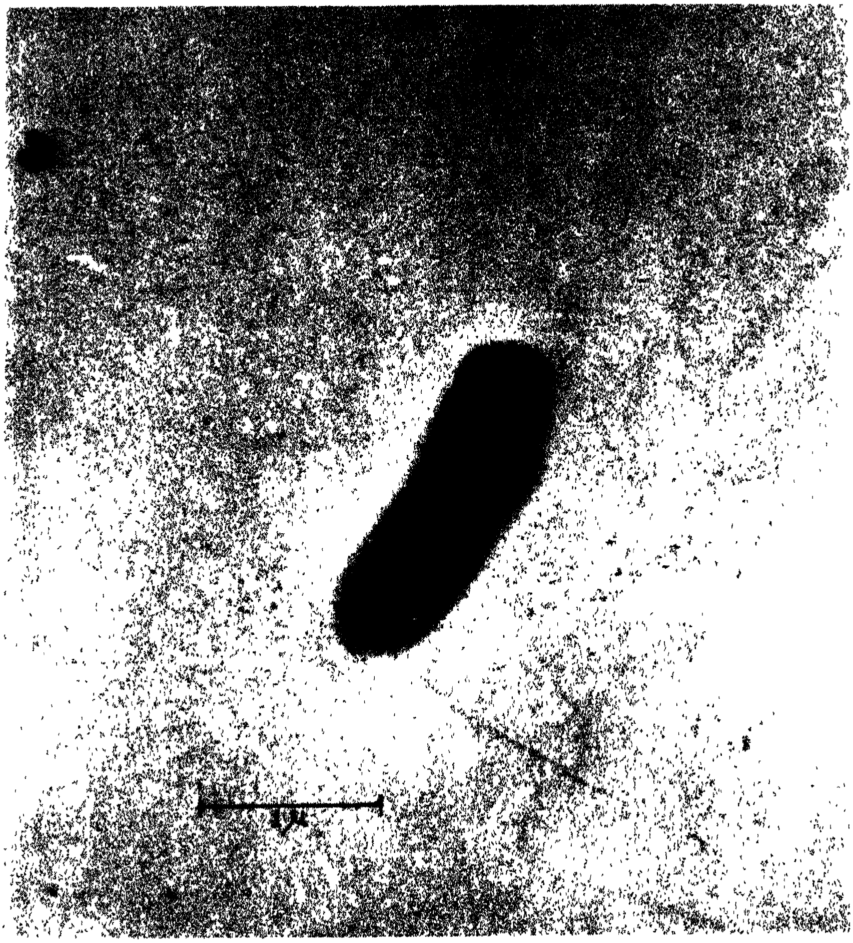

Fig. 1. Electron Micrograph of Cell of Rhizobium leguminosarum, Showing Flagellum

Preliminary observations. During the course of some work with the electron microscope on *Azotobacter* flagellation during the summer of 1943, there was opportunity for a limited amount of study, also, of some preparations of bacteriophage for *Rhizobium leguminosarum*, the pea nodule organism. These studies were conducted through the courtesy of Dr. James Hillier and the RCA Laboratories at Princeton, New Jersey.

Cells of *Rhizobium leguminosarum* from the sauerkraut calcium-glycerophosphate medium of Campbell and Hofer (1943) appeared as in figure 1, and figure 2 illustrates the appearance of the cells in the presence of the bacteriophage. In figure 2 can be seen particles like those said by Luria, Delbruck, and Anderson (1943) and by Baylor, Severens, and Clark (1944) to be bacteriophage. There are also smaller particles of the same shape, and still smaller particles that appear to be round. Study of figure 2 shows that the diameter of the particles and, particularly, of the larger ones, is greater than that of the bacterial flagellum which is visible in the lower left-hand corner of the picture. The same thing was found to be true in the published plates of Luria, Delbrück, and Anderson (1943).

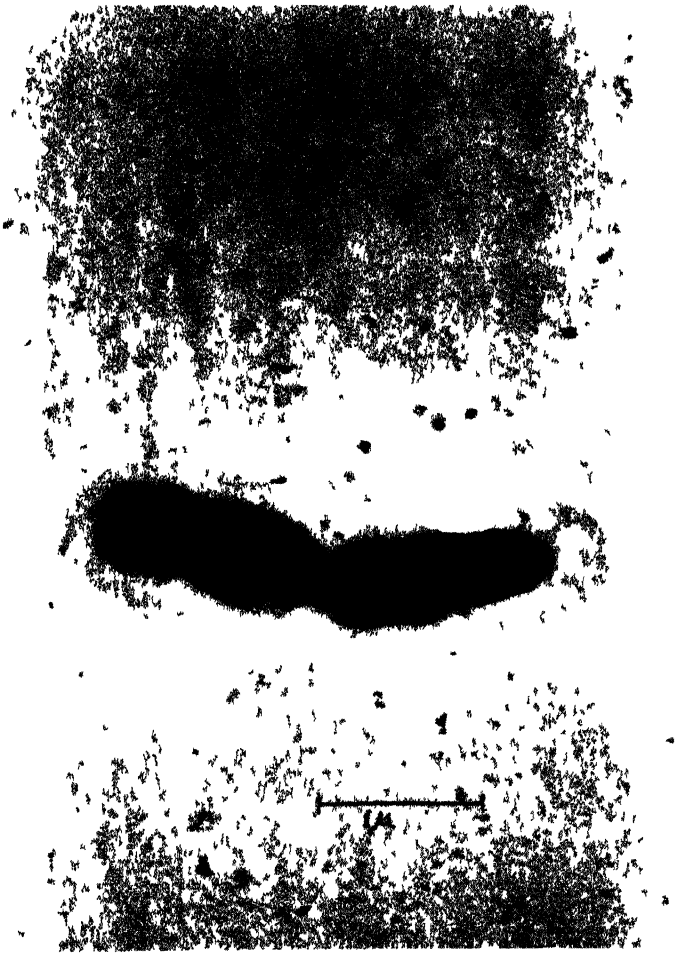

FIG. 2. ELECTRON MICROGRAPH OF DIVIDING CELLS OF RHIZOBIUM LEGUMINOSARUM SHOWING ATTACHED FLAGELLUM AND, IN VICINITY, BACTERIOPHAGE PARTICLES

This suggested that, since bacterial flagella can be seen under the ordinary microscope, following staining by appropriate technique, these particles, which have a still greater diameter, should also be visible. This is all the more true since the fact has been shown that the larger viruses can be stained and examined under the ordinary microscope (for reviews of procedures see Kaiser, 1938; Mallory, 1938; van Rooyen and Rhodes, 1940). Furthermore, Burnet and Andrewes (1933) make a similar statement, not only for certain viruses, but for the larger bacteriophages. (After the present work was begun, Pijper, in 1945, reported that the bacteriophage of *Eberthella typhosum* could be seen under the ordinary microscope by dark-field studies, using sunlight as the source of illumination. The present work has been reported in a preliminary way by Hofer and Richards in 1945.)

Objects. In view of the above possibilities, it seemed worth while to try to find procedures by which bacteriophage could be seen under the ordinary microscope. Another purpose was to attempt, thereby, to bring greater simplicity and directness into the study of bacteriophage than is possible by the use of the electron microscope. Furthermore, staining procedures, if they could be developed, would be helpful in differentiating various kinds of material.

STAINING METHODS

Use of fluorescent dyes. Work by Richards, Klein, and Leach (1941) with the tubercle organism shows that the cells appear larger when stained with carbol auramin and irradiated with ultraviolet. Cells so stained convert the invisible short waves into the longer waves of visible light. There are reports that the same principle can be used to magnify the images of the virus particles (Hagemann, 1937; Kaiser, 1938; Levaditi and Reinie, 1939; Aksel, 1941; Gohde, 1941), and this suggested the use of this technique for the particles under investigation.

Since the procedures with carbol auramin had been well worked out for use in the diagnosis of tuberculosis, the first work was done with this dye. Tests were made simultaneously, however, with primulin and thioflavine. Various procedures were investigated for use with these dyes, but the one that seemed best and was used extensively with carbol auramin is as follows:

Place a pyrex slide in cleaning solution 10 minutes; wash thoroughly in running water; leave in alcohol 10 minutes; flame thoroughly; and cool.

Add 3 loopfuls of sterile distilled water, 1 loopful of bacteriophage suspension, and 1 loopful of pea nodule organism, using a 24-hr culture incubated at 30 C in the sauerkraut-calcium-glycerophosphate medium of Campbell and Hofer (1943).

Spread over surface of slide and let stand to dry.

Before the slide is completely dry, add another portion of bacteria similar to the one described above, and spread.

Allow to dry.

Flood slide with carbol auramin (auramin, 0.3 g; phenol, 3.0 g; distilled water, 100 ml) and allow to stand for 10 minutes.

Blot dry.

A more concentrated solution of carbol auramin has sometimes been substituted for the one above (0.25 g auramin; 1.0 g phenol; 10 ml distilled water).

Examination of the preparation is made by the use of an H-4 mercury arc as the source of ultraviolet and an ordinary microscope fitted with an aluminum reflector instead of the usual mirror. No filter was used, as it was not known in what color the particles might fluoresce, and use of a filter would exclude the rays of colors other than that of the filter.

Modified acid-fast stain. For observing the particles by ordinary light, a number of different stains were tested. One of the most helpful procedures was a modification of the ordinary acid-fast stain commonly used for the tubercle organism. The particular technique that was finally developed resulted from application of the results of Henry and Stacey (1943) and Bartholomew and Umbreit (1944), who reported that organisms which retain the crystal violet of the gram stain are able to do this because of the presence in the cell wall of the magnesium salt of ribonucleic acid. Researches of that time (Northrop, 1938; Hoagland, 1943; Bawden, 1945) indicated that virus protein contains a comparatively high proportion of this compound. Since, among bacteria, acid-fast strains are gram-positive, it seemed that acid fastness might be associated with this characteristic. At any rate, it seemed worth while to find whether techniques which favored retention of the crystal violet of the gram stain would act the same way for acid-fast particles. Because of the extremely small size of these, it was necessary to devise a solution which would decolorize the relatively large bacterial cells, but not the extremely small particles. For this reason, in the development of the decolorizing solution, when the proper quantities of alcohol, water, and hydrochloric acid had been determined, varying quantities of magnesium were added. As suggested above, the presence of this element in the solution did seem to exert a protective action upon the dye in the particles during the process of decolorization.

The final procedure that was adopted is as follows:

Prepare slide with distilled water, bacteria, and bacteriophage as for the auramin stain above.

Stain 10 minutes with Ziehl's carbol fuchsin (10 ml saturated alcoholic basic fuchsin and 90 ml of 5 per cent phenol).

Wash gently with running water.

Dip directly into, and agitate gently in, a decolorizing solution (consisting of equal parts of alcohol and distilled water with sufficient HCl to make an N/5 solution of the mixture, and $MgCl_2$ sufficient to make an N/10 solution of Mg); remove after 3 to 5 seconds.

Agitate gently in distilled water in a staining jar for 3 to 5 seconds.

Remove and, without drying, flood with sufficient 1 per cent aqueous methylene blue to replace the excess water. Allow to act for 20 seconds, wash in running water, blot dry, and examine.

The addition of the cells just before the drying of the preparation was helpful for observation of the bacteria since it provided whole cells in which the lytic process had not begun, visibly, to affect the structure. Consequently, these could be distinguished sharply from the other material on the slide, and their presence gave a better indication of how the stain was working.

Modified flagella stain. In the above procedure, acid and alcohol were used to decolorize, and the acid-fast compounds were evidently at the same time protected by the presence in the decolorizing solution of magnesium. Similar variations of the Hofer and Wilson (1938) flagella stain were attempted. The most suitable procedure proved to be the following:

Prepare slide with bacteria, bacteriophage, and water, as described.

Mix 5 ml saturated, aqueous potassium alum, 2 ml saturated, aqueous mercuric chloride, 2 ml 20 per cent tannic acid, and 0.4 ml saturated alcoholic basic fuchsin. Filter through a double thickness of filter paper. Apply to slide for 8 to 10 minutes.

Wash in running water and dry in air.

Stain 5 minutes with Ziehl's carbol fuchsin.

Wash gently with running water.

Place directly, with gentle agitation, into decolorizing solution (as above, but without magnesium) for 3 to 5 seconds.

Place in a staining jar of distilled water and agitate gently for 3 to 5 seconds.

Remove and flood with 1 per cent aqueous methylene blue sufficient to wash off the water, and allow the dye to act for 20 seconds.

Wash in running water and blot dry.

STAINING RESULTS

Carbol auramin. The first tests of the carbol auramin stain were made in December of 1943 and January of 1944. It was found that in aqueous solution auramin alone was not suitable, and that if even small amounts of alcohol were used the results were not satisfactory. Even washing with water tended to weaken the glow of the particles. Only in the presence of carbol auramin, used as described above, was it possible to see bright particles, usually occurring singly, but sometimes attached to the cells or inside them.

Under the ordinary microscope, with the preparation irradiated by ultraviolet, these appeared as innumerable tiny, glowing points. Some were large enough to be seen plainly, but others were barely visible. The preparation appeared much like the heavens on a very dark night, with some stars appearing bright and others only weak. In contrast to this, the bacteria on the slide appeared as very dim, hardly visible, lemon-yellow bodies without any brilliance whatsoever, except as one of the bright points might happen to be attached to a cell or as it might develop some acid fastness of its own during the lytic process.

While auramin is the only fluorescent dye so far found to yield such results, it has given them many times. Although stains with auramin do not always result in the demonstration of these bodies, it is still true that good results are frequent, and that the best preparations are made with freshly prepared auramin solution.

There are two advantages of this procedure. One is the simple method for staining the preparation, and the other is the ease of demonstrating the bodies. Even though a well-darkened room is necessary for demonstration, when the eyes are adjusted to this condition, the glow of the tiny particles can be seen distinctly. Disadvantages are that some slides fail to show the presence of particles even when they are present, and that some strains of bacteria, even without added bacteriophage, show these glowing points.

Modified acid-fast stain. Preparations of cells undergoing lysis, stained with Ziehl's carbol fuchsin, decolorized by the method given above, and counterstained with methylene blue, showed, when examined by the ordinary daylight lamp, four types of material. There were blue cells of bacteria, red particles, brownish yellow partially lysed bacterial protoplasm, and structures whose identity is not known.

The cells, before the lytic process had begun, were regular in shape, as shown in figure 3a, and they were usually blue. The color tended to vary somewhat, however, depending apparently upon the severity of the decolorizing process. Too long a decolorization tended to remove the dye from the particles, and too little decolorizing left the cells with some red color.

The particles increased in size in the presence of living bacteria, and they stained particularly sharply following their formation in the newly lysed cell. By the use of a slide having many kinds of material on it, a diversity of acid-fast particles became apparent. Though many of the red particles were of the same shape as those shown under the electron microscope (appearing like dumbbells cut in two through the middle of the handle), a greater number appeared round. This may have been due to position, since the tail is only visible when extended horizontally, but there is the possibility that some particles may not have a tail. This is particularly true of the smaller ones. The particles ranged from the largest acid-fast ones, which were fully half a micron in size, to the smallest particles, which appeared to be about one-tenth that size, or even smaller. All were numerous, acid-fast, and easily seen; furthermore, they were practically never seen on preparations made from bacteria alone. It is only when bacteriophage is added and the culture allowed to stand a while that a great profusion of very small, acid-fast bodies is seen.

An interesting point in connection with the staining of these bodies is the fact that cells can seldom be seen with particles attached, as they appear under the electron microscope. When these are seen, as often as not the particles will not be acid-fast but, rather, blue, like the cell. On the assumption that these particles actually are the bacteriophage particles, it is possible that upon attachment to the cell there is an interchange of protoplasm, with the result that some of the acid-fast material enters the cell, and some of the cell protoplasm enters the bacteriophage particle. It is interesting that both d'Herelle and Pijper failed to see particles attached to the outside of the cell as these appear under the electron microscope. Apparently, the ordinary microscope does not show these structures well, or they may have been induced in greater numbers than usual by the centrifugation to which the bacteriophage was subjected before examination under the electron microscope.

The modified acid-fast stain possesses a great advantage, however; although preparations vary in quality, the results are usually sufficiently satisfactory to show detail not evident by any other method now at hand. Photographs taken of material stained by this method are shown in figure 3. These show normal cells, the particles under investigation, and cells that are progressively to a greater extent destroyed by the action of the lytic agent. Another advantage of the method is that, although, in general, it shows structures like those evident under the electron microscope, these differ in color and can, therefore, be differentiated one from another. The only disadvantage is that the color differentiation is not always as sharp as it should be and that the cells sometimes appear smaller than they do by other techniques.

Modified flagella stain. For purposes other than research, or for the beginner in the field, this is the best stain. Even for research, it occasionally gives results comparable to those obtained with the acid-fast technique. While the procedure is somewhat complicated to use, the results are uniformly good. The colors are much like those in the acid-fast stain, but the differentiation among the different kinds of material is sharp. There is no running together of colors as in the acid-fast stain, the cells appear large and blue, and the particles are large and red. The great disadvantage of the technique is that it is only rarely that the smaller particles are visible. For demonstration purposes, however, this feature is advantageous.

PHASE MICROSCOPE STUDY

There remained one question is regard to this work, namely, whether the particles seen through the microscope or shown by the electron microscope are actually bacteriophage. Despite the fact that from all indications they were bacteriophage and that there was no other satisfactory explanation, there was no proof that these bodies were bacteriophage. Evidence concerning this point was obtained through the use of the phase microscope recently developed by the American Optical Company of Buffalo, New York, and kindly loaned to the author by Mr. A. H. Bennett of that company.

By use of this instrument for the examination of living bacteria it was seen that the characteristic particles mentioned above bring about certain changes in the bacteria studied that result in destruction of the cells. Furthermore, in some cases, individual particles could be seen to become attached to individual cells, to develop thereon, and finally to destroy the cells. In one case in which pho-

FIG. 3. PHOTOGRAPHS OF BACTERIA AND BACTERIOPHAGE, STAINED BY THE MODIFIED ACID-FAST METHOD, AS SEEN THROUGH THE ORDINARY MICROSCOPE. × 1,700

(Photographs obtained through the co-operation of Dr. O. W. Richards, of the American Optical Co.)

A. Cells of *Rhizobium leguminosarum*, the pea nodule organism.

B. Particles of bacteriophage.

C. Bacteriophage particles attached to cells.

D. Bacteriophage particles attached to cells, with appearance of abnormalities in structure due to the action of bacteriophage.

E. Partially lysed cells showing development of new bacteriophage particles.

F. Cells in which the lytic process is well advanced (in two of the cells the original bacteriophage particle to make contact with the cell is still attached).

G. Outlines of old cells in which are seen the newly formed acid-fast bacteriophage particles.

H. Structures of unknown origin and function which are frequently seen.

I. Acid-fast particles remaining after lysis of the cells.

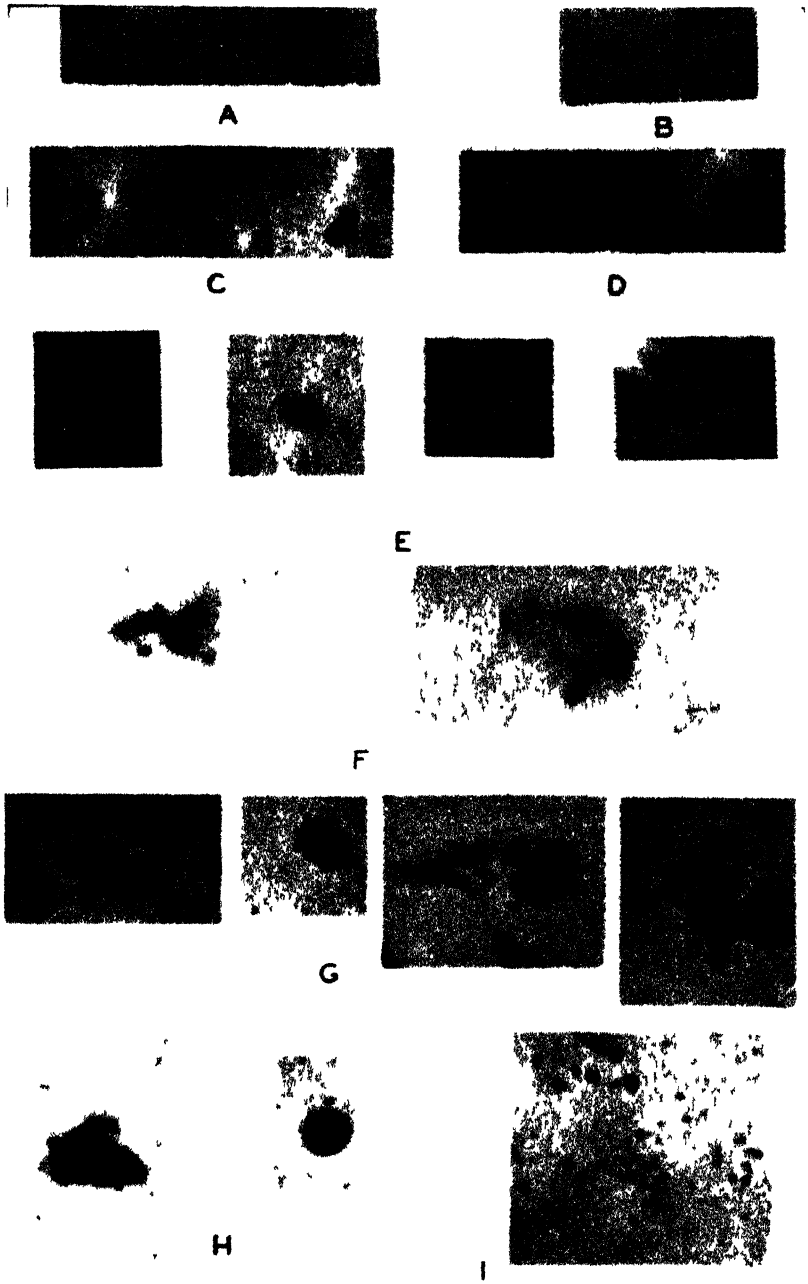

Fig. 3

tographs were taken, the particle when first seen was 4 or 5 microns from the cell. The two united; the particle appeared to become smaller with a long, slender attachment to the cell; and for a period of perhaps 20 minutes there seemed to be an interchange of material between the two. Then the particle enlarged many times, and this process of growth continued until the bacterial cell wall burst and protoplasm oozed out of the cell to such an extent that the particle was hidden from view. Even then the protoplasm continued to flow until apparently all that was in the cell had been lost.

Following observations of this nature, it was possible to re-examine stained slides and to see on them structures which had not been noticed previously, or which had been seen but not recognized. These were characteristic of the different stages through which the cell and its attached bacteriophage may pass during the process of lysis, and were clearly visible in the stained preparations.

As a result of the work with the phase microscope, therefore, it is evident that the particles that are present in suspensions of bacteriophage for *Rhizobium leguminosarum*, and that can be seen under the electron microscope or in suitably stained preparations, are bacteriophage, as shown by the fact that they do lyse bacteria.

SUMMARY

Experiments were begun (1) to find whether the small particles present in bacteriophage preparations and shown by the electron microscope could be stained and made visible under the ordinary microscope, and (2) to learn, if possible, whether these particles are, indeed, bacteriophage. The work of the author and of Luria, Delbrück, and Anderson (1943) showed that these particles have a diameter greater than that of bacterial flagella.

Bacteria for inoculating peas and bacteriophages specific for these bacteria were used in these investigations.

Three staining methods, each having its own advantages and disadvantages, were developed for demonstrating these particles under the ordinary microscope: (1) staining with carbol auramin and examining under ultraviolet irradiation, (2) a modification of the Ziehl-Nielsen acid-fast stain, and (3) a modification of the Hofer and Wilson (1938) flagella stain.

It has been shown that the particles in question are ordinarily acid-fast.

By staining preparations of various ages, it became apparent that the particles enlarge in size in the presence of bacteria; that they increase in number as the bacterial cells decrease; and that the particles developing in the partially lysed cells and showing in the zones where bacterial cells have been before lysis has occurred stain particularly sharply.

Using the newly developed phase microscope, it has proved possible to see the same particles unstained while they are still in living condition. In this way, it is possible to see and to photograph the successive stages of lysis; the immobilization of the cells; the appearance of the larger, i.e., visible, bacteriophage particles; union of these with the cells; the preliminary diminishing of the size of the granules subsequent to attachment to the cells; and, immediately fol-

lowing, the increase in size of the former at the expense of the cells, until the latter burst and the protoplasm flows out. Finally, there is development within the cell protoplasm of new bodies similar to the original granules. These observations seem to demonstrate that the particles under observation actually are those of bacteriophage.

Furthermore, after observing under the phase microscope the various stages of lysis, it was possible to re-examine under the ordinary microscope preparations made by the acid-fast technique and to locate therein structures that had previously been seen only under the phase microscope. This demonstrates both the existence of the bacteriophage particles and the usefulness of the ordinary microscope for seeing them.

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GLYCERITE OF HYDROGEN PEROXIDE

I. Comparison of Its Bacteriotoxic Action with That of Mercurial Solutions

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A new modification (a glycerite) of an old antiseptic, hydrogen peroxide, was described by Brown, Krabek, and Skiffington (1946) with the results of some preliminary tests of antiseptic action. The glycerite of hydrogen peroxide is derived from a solution of urea peroxide (4 per cent) in substantially anhydrous glycerol with 8-hydroxyquinoline (0.1 per cent) as an added stabilizer. A solution which, for practical purposes, is similar, may be made by dissolving pure, relatively nonaqueous hydrogen peroxide (92 per cent) in glycerol with 8-hydroxyquinoline (oxine) and with or without added urea. It was considered desirable to amplify these preliminary bacteriological findings with an extended comparison with a number of the more common antiseptics readily available commercially, in order to determine how the newer antiseptics, together with control solutions, compared with these well-known solutions. The results of the comparison of the new antiseptic solution with a number of organic mercurial solutions are reported in this paper.

The cup plate technique has been suggested by Ruehle and Brewer (1931) as a method for testing substances intended for continued application to tissue. Although the method is by no means a perfect test for antiseptic action under clinical conditions, it does give some information not so readily obtainable by other means concerning the bacteriotoxic action of a solution when continually applied. Because of errors, resulting from the testing of certain antiseptic solutions while using the FDA technique, it was found necessary to evolve a method utilizing the same principle but using paraffined cylinders instead of the open cup. A more complete description of the method, together with some of its advantages and disadvantages, is being submitted in another paper.

As Nye (1937) and others have pointed out, some antiseptic solutions, as available in commercial strengths, cannot be used undiluted because of either irritation to tissue or other undesirable effects. The peroxide solutions reported here have been used undiluted on extensive lesions, with little or no undesirable reaction. This phase of the work has been reported upon by Brown (1946*a*) and expressed or implied in other papers on the application of the peroxide-glycerol solutions in various pathological conditions. All solutions were used in the tests undiluted, in order to observe the maximum effect that may be obtained.

The physiological properties of the peroxide-glycerol solutions have been described by Brown (1946*b*, 1946*c*). The properties of the mercurial solutions have been summarized by McCulloch (1945).

The solutions tested comprised the following:

Urea peroxide (4 per cent) in glycerol.

Urea peroxide (4 per cent), 8-hydroxyquinoline (0.1 per cent) in glycerol (glycerite of hydrogen peroxide).

Hydrogen peroxide (1.46 per cent) in glycerol (corresponding to a 4 per cent urea peroxide solution).

Hydrogen peroxide (1.46 per cent), 8-hydroxyquinoline (0.1 per cent) in glycerol.

Merbromin (N. F), surgical 2 per cent and solution (aqueous) 2 per cent.

Merseptal, tincture, 1:500, and aqueous, 1:1500.

Mercurochrome, surgical 2 per cent, and solution (aqueous) 2 per cent.

Mercresin, tincture, 1:1,000.

Merthiolate, tincture, 1:1,000, and aqueous, 1:1,000.

Metaphen, tincture, 1:200, and aqueous, 1:500.

The controls consisted of "tincture solvent" (ethyl alcohol 50 per cent; acetone 10 per cent; water 40 per cent), glycerol, glycerol saturated with urea, and 8-hydroxyquinoline (oxine) 0.1 per cent in glycerol. Under the conditions of the test, the control solutions, with the exception of the oxine in glycerol, showed negligible effect, as indicated by a failure to demonstrate a zone. The results with the oxine solution are shown in the tables.

The following bacterial species are reported upon in this paper: *Staphylococcus aureus* (FDA209); *Staphylococcus albus*, coagulase-positive; *Staphylococcus pharyngis; Streptococcus liquefaciens*, alpha hemolytic; a diphtheroid; *Bacillus subtilis; Escherichia coli*, var. *communior; Proteus mirabilis; Pseudomonas aeruginosa*, and *Aerobacter cloacae*. The organisms were, with the exception of *Staphylococcus aureus* (FDA209) which came from the Food and Drug Administration, strains freshly isolated from lesions of clinical interest. Other strains of organisms and of streptococci and staphylococci, in particular, were examined. Only one of each is included here, since the others gave essentially the same results, although the clinical strains of *Staphylococcus aureus* tended to be slightly more resistant to some of the mercurial solutions. It is intended to report, at a later time, a statistical examination of the results obtained with a number of strains of different organisms as affected by different antiseptics. All organisms demonstrated the characteristic biological reactions as given by Bergey *et al.* (1939). The *Bacillus subtilis* strain used is apparently not a very resistant strain, since solutions which show little or no action against other bacteria show a definite zone with this strain.

A cylinder agar plate method was used, since its prototype, the agar cup plate, has been suggested as a method for testing substances used for prolonged clinical application. Harris and Prout (1940) considered that this type of method gave a somewhat better correlation between *in vitro* and *in vivo* applications than some other methods, although there is some disagreement on this score. The method was essentially as follows:

Difco glucose agar, with or without 10 per cent added horse serum, was preinoculated with 0.2 ml of an undiluted 22- to 26-hour glucose broth culture of the organism, for each 25 ml of melted agar. The inoculated agar was then dispensed in 25-ml amounts into sterile 100-mm petri dishes with unglazed clay covers. After hardening of the agar, slightly warmed "penicylinders" of 8-mm outside diameter, the rims of which had been paraffined, were placed on the surface of the agar. The solution to be tested was then pipetted in 0.2-ml amounts into the cylinders with a sterile pipette. The plates were immediately placed in the incubator at 37 C and incubated for 18 hours. At the end of this time, the plates were removed and subcultures of approximately 3 mm in cross section were taken at intervals of 1 to 3 mm radially and placed in modified Brewer's thioglycolate medium (Baltimore Biological Laboratories, list 135). The subculture tubes were then incubated at 37 C until growth occurred, or for at least 7 days. Before the subcultures were discarded, those tubes showing no growth were reinoculated with a small inoculum (50 organisms or fewer) of the same strain tested and incubated for 3 days, or less if growth occurred sooner. This technique determined that the test solution was not carried over into the subculture tubes in amounts that were inhibitive to growth of normal bacteria.

After subculturing, the distance of the outer edge of the clear zone (if any) from the outer edge of the cylinder was measured to the nearest 0.5 mm and recorded for each test. At the same time, the distance from the edge of the cylinder of the inner and outer edge of the indentation left by the removal of materials for subculturing was measured and recorded. Because of space limitations, the tables show only the average distal measurements of the subculture area closest to the cup that showed growth in the subculture tube. This obviously favors the antiseptic. At least three, and in some cases more, replicate tests were made with each antiseptic solution.

The values in the tables have been rounded off to the nearest whole number, since this is sufficient for comparative purposes. Table 1 lists the zones obtained with gram-positive organisms, and table 2 those obtained with gram-negative organisms. The first column (A) indicates the apparent visible zone, and the second column (B) the zone as determined by subcultures for plates without serum. The corresponding values for plates containing 10 per cent horse serum are given in columns (C) and (D). In addition to the visible changes as shown by the zones, there are a number of miscellaneous observations which may be of interest in relation to the possible modes of action of the antiseptics on the organisms tested. These are given briefly, in order that the record may be complete.

Opaque zones within the zones of apparent inhibition were noted in the case of the following: surgical merbromin with *S. pharyngis* and *E. coli* in serum plates; aqueous merbromin with staphylococci in both serum and plain plates; surgical mercurochrome with some organisms, particularly the staphylococci. Control plates without organisms show no opaque zones, either with or without serum. The opacity may be due to acid formation by the organism with concomitant precipitation of constituents of the solution.

Increased absorption of pigment at the edge of the zone of inhibition was shown by *Aerobacter cloacae* with surgical merbromin and by *P. aeruginosa* with aqueous merbromin, tincture of mercresin, surgical mercurochrome, tincture of merseptal, and aqueous merbromin. Increased colony size distal to the area of inhibition was shown by *P. aeruginosa* with aqueous merbromin and tincture of metaphen, and by *Proteus mirabilis* with tincture of Merthiolate. Raised wet growth (mucoid) appeared in the case of *Aerobacter cloacae* with surgical merbromin and glycerite of merthiolate, and with *B. subtilis* with surgical mercurochrome.

TABLE 1

Comparison of action of glycerite of hydrogen peroxide and mercurial antiseptic solutions on gram-positive organisms

GLYCEROL SOLUTIONS		S. AUREUS				S. ALBUS				S. PHARYNGIS				S. LIQUEFACIENS				DIPHTHEROID				B. SUBTILIS			
		A[*]	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
Urea peroxide + oxine	G[†]	13	13	13	11	19	20	14	13	18	10	9	12	11	15	8	12	16	12	14	11	13	13	11	11
Urea peroxide	G	14	15	13	10	18	18	11	12	12	12	9	9	10	8	8	8	15	13	17	5	13	13	7	7
Hydrogen peroxide + oxine	G	13	15	17	9	22	23	20	17	12	15	7	12	11	14	9	10	9	12	5	5	13	13	11	14 6 6
Hydrogen peroxide	G	13	10	6	8	18	18	11	12	10	4	7	7	12	13	6	5	12	13	9	6	14	13	9	4
Oxine alone	G	10	6	7	7	15	14	10	11	5	4	2	3	6	3	5	2	16	8	6	5	15	16	18	10
MERCURIAL SOLUTIONS																									
Merbromin	T	12	16	10	12	15	15	10	13	6	5	6	6	8	5	6	5	18	13	8	13	15	17	11	13
	A	6	11	5	4	7	5	3	5	4	4	4	4	6	5	2	2	8	8	6	5	9	12	5	9
Mercresin	T	11	10	7	8	14	13	7	10	9	5	6	4	7	6	5	5	16	15	10	13	15	15	12	12
Mercurochrome	T	10	9	8	11	14	13	7	8	8	5	5	4	6	3	5	5	11	13	6	8	10	13	7	10
	A	6	5	9	7	8	6	6	8	8	4	3	4	6	4	3	2	8	9	5	8	8	11	5	8
Merseptal	T	12	8	7	4	16	15	9	7	10	5	5	4	9	6	6	4	20	14	9	11	15	16	11	10
	A	2	4	8	4	17	16	9	9	10	3	5	3	9	5	5	3	13	14	7	11	15	15	9	8
Methiolate	T	16	11	10	6	21	15	11	8	9	7	6	5	8	5	5	5	25	13	7	8	23	17	18	15
	A	16	4	10	3	21	9	12	3	9	3	6	3	9	4	7	3	19	5	11	15	17	15	11	11
	G	15	5	8	3	17	7	10	3	7	3	3	3	8	3	1	3	15	4	9	3	13	7	7	6
Metaphen	T	10	14	9	6	12	11	8	8	6	8	6	5	8	10	7	7	11	12	6	11	14	15	11	12
	A	10	5	5	5	13	12	7	7	9	3	4	3	8	6	1	3	11	13	7	9	11	13	6	6

Zones were measured radially, in mm, from the edge of cylinder to the edge of clear zone.

[*] Column headings: A = apparent visible zone of inhibition—no serum; B = zone as determined by subculture—no serum; C = apparent visible zone of inhibition—10% serum; D = zone as determined by subculture—10% serum.

[†] Abbreviations: T = tincture; A = aqueous; G = glycerol.

Changes in pigmentation were observed with *P. aeruginosa*, normally a blue-green culture, with the following: surgical merbromin showed an increased production of pigment; aqueous merthiolate produced a 2-mm zone of decreased pigmentation; glycerite of merthiolate caused a 6-mm pink zone; tincture of metaphen showed a 3-mm yellowish zone followed by a 4-mm pink zone distal to the yellow zone.

The glycerol solution of the urea peroxide and oxine resulted in a zone of raised wet mucoid growth in the case of *Aerobacter cloacae*. The organisms within the zone were coccoid in nature instead of the normal, small, rodlike shape. In one series of subcultures they maintained this characteristic for several transfers. With the same solution *P. aeruginosa* showed a reaction to the antiseptic by turning from a normal bluish-green culture to an emerald-green in plates without serum. In plates with serum, there was a yellow zone, followed by an emerald zone extending peripherally from the cup.

TABLE 2

Comparison of action of glycerite of hydrogen peroxide and mercurial antiseptic solutions on gram-negative organisms

GLYCEROL SOLUTIONS		E. COLI				P. MIRABILIS				P. AERUGINOSA				AEROBACTER CLOACAE			
		A*	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
Urea peroxide + oxine	G*	7	6	6	7	10	9	7	7	5	3	4	4	8	10	8	8
Urea peroxide	G	5	5	7	5	7	6	7	6	4	3	4	5	6	6	6	9
Hydrogen peroxide + oxine	G	4	5	3	3	7	6	6	3	5	3	4	3	6	6	7	7
Hydrogen peroxide	G	3	3	7	6	5	3	5	6	1	2	1	3	6	6	6	6
Oxine	G	4	3	0	0	7	3	2	3	0	3	0	3	6	5	0	3
MERCURIAL SOLUTIONS																	
Merbromin	T	6	18	5	13	8	18	7	16	10	14	7	13	15	15	14	14
	A	4	7	3	4	6	6	3	7	5	7	4	9	3	7	4	6
Mercresin	T	4	10	1	7	8	9	5	6	5	9	5	8	7	14	8	8
Mercurochrome	T	6	14	3	11	7	7	6	8	9	10	6	9	6	12	8	9
	A	3	5	2	3	6	8	4	8	5	7	5	6	3	7	4	6
Merseptal	T	6	8	3	8	9	6	6	7	7	11	6	7	7	7	6	5
	A	6	10	2	3	5	5	4	4	5	6	3	5	6	5	4	3
Merthiolate	T	7	13	5	11	11	12	7	6	9	13	9	9	13	13	14	15
	A	6	6	3	5	6	7	6	3	10	8	7	10	11	11	8	10
	G	5	4	4	3	5	5	4	3	9	5	7	6	6	6	6	7
Metaphen	T	7	13	5	11	9	9	8	17	10	14	7	10	7	14	11	15
	A	2	3	1	3	4	3	4	3	7	4	5	5	3	2	2	3

Zones were measured radially, in mm, from the edge of cylinder to edge of clear zone.

* See footnotes to table 1.

Urea peroxide without oxine caused a deeper pigmentation in *S. aureus* cultures, with an undercutting of the clear zone. In *Proteus mirabilis* cultures, there was a raised moist growth immediately distal to the clear zone. Hydrogen peroxide with oxine, in glycerol, caused a pink zone immediately distal to the clear zone with *P. aeruginosa*, the remainder of the plate remaining an emerald green.

The peroxide solutions evidently showed greater activity than did the mercurial solutions against gram-positive organisms. The addition of oxine results in somewhat greater activity of the solutions in the presence of serum, but not much difference in the absence of serum. The values resulting from oxine alone are normally overshadowed by the effects of the hydrogen peroxide. In addition, slightly more bacteriostatic action, as indicated by decreasing density of the colonies, is shown by solutions containing oxine as compared with the solutions not containing the stabilizing agent. The solutions of hydrogen peroxide in glycerol, without urea, demonstrate somewhat less activity than did the solutions with urea. It will be noted that there is apparently more effect on the bacterial metabolism, as shown by variations in the type of growth, by the solutions containing oxine and urea than with hydrogen peroxide alone in glycerol.

The alcoholic mercurial solutions showed better activity than did the aqueous ones. Some of the tinctures showed larger zones against the gram-negative organisms tested than do the peroxides. The latter, however, showed as good or better results when compared to the aqueous solutions of the mercurials. Thus, since the alcoholic solutions cannot be used on large wound areas because of the irritation, the glycerite of hydrogen peroxide would seem more applicable in such cases. Another disadvantage suffered by the mercurial solutions is the undesirability of the risk of absorption of mercury compounds. Such is not the case with the peroxide solutions of constituents which are nontoxic and nonallergenic. Tissue tolerance studies have shown them to be nonirritating on both normal and infected skin and mucous membranes (Brown, 1946*a*).

The solutions of merbromin, in general, give consistently larger zones than do mercurochrome solutions. The cause of this is not immediately apparent, since the two are supposedly of the same concentration and of essentially the same composition. The answer may lie, however, in the pH of the two solutions, since the apparent pH of the surgical mercurochrome was 9.6, whereas that of the corresponding merbromin solution was 8.1. The fact that both solutions show as good results as they do on the plates may be due to the long-continued action, since McCulloch's (1945) review indicates that, as ordinarily applied clinically, the solutions leave much to be desired.

SUMMARY

When tested by a modified cylinder plate method, peroxide-glycerol solutions, made from either urea peroxide or hydrogen peroxide, showed a bacteriotoxic action on both gram-positive and gram-negative organisms.

A greater bactericidal effect was noted with gram-positive than with gram-negative bacteria.

The presence of 8-hydroxyquinoline in the peroxide solution did not appear to improve significantly the bacteriotoxic action of the solution in the absence of serum, but did appear to enhance the activity in the presence of 10 per cent horse serum.

In comparison with 12 mercurial solutions, the glycerol-peroxide solutions showed, in general, greater bacteriotoxic action on gram-positive organisms than did the mercurial solutions. The latter were, in general, the more effective on gram-negative bacteria. In specific cases, however, the peroxide-glycerol solutions were more efficacious than some of the mercurial solutions, particularly when water was the principal solvent for the mercurial compound.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

INDIANA BRANCH

INDIANAPOLIS, INDIANA, FEBRUARY 21, 1947

POSSIBLE IDENTITY OF THE HEMOLYSIN OF CLOSTRIDIUM NOVYI, TYPE B, WITH LYSOLECITHIN.

R. C. Bard and L. S. McClung, Indiana University, Bacteriological Laboratories, Bloomington, Indiana.

Preliminary study of the chemical nature of the toxin of *Clostridium novyi*, type B, indicates that the hemolytic substance is lysolecithin. This is based upon the inhibition by lecithinase B (the specific enzyme catalyzing the hydrolysis of lysolecithin) of the hemolytic action of the toxin of an authentic strain of *C. novyi*, type B. Lecithinase B was prepared from old rice bran according to the method of Contardi and Ercoli. Lysolecithin was prepared by permitting dehydrated rattlesnake venom to react with purified lecithin obtained from soy beans. The rice bran enzyme completely destroyed the hemolytic activity of the lysolecithin and markedly inhibited the hemolytic activity of culture supernatants of *C. novyi*, type B, as well as desiccated toxin prepared by salting out with ammonium sulfate. This was not wholly unexpected in view of the general lecithinase activity of other clostridial toxins.

THE LONGEVITY OF BACTERIAL CULTURES UNDER PARAFFIN OIL.

S. E. Hartsell, Purdue University, Lafayette, Indiana.

The use of paraffin oil for the preservation of 242 strains of bacteria was studied. Giant colony growths on stab cultures of yeast water, veal infusion agar (5 per cent rabbit blood added for hemophilic species) were layered with sterile paraffin oil and held at 25 C. The viability was tested at intervals for 63 months using media of the same type employed for the preservation of the culture. The genera, number of species, number of strains, and maximum survival time (in months) when last tested were as follows: *Achromobacter*, 19, 33, 51; *Aerobacter*, 1, 3, 46; *Alkaligenes*, 2, 2, 46; *Bacillus*, 10, 40, 48; *Brucella*, 2, 2, 57; *Eberthella*, 1, 3, 36; *Escherichia*, 1, 53, 63; *Flavobacterium*, 8, 15, 48; *Gaffkya*, 1, 3, 46; *Hemophilus*, 1, 1, 57; *Leuconostoc*, 1, 5, 31; *Microbacterium*, 1, 1, 45; *Micrococcus*, 10, 19, 46; *Pasteurella*, 1, 1, 57; *Proteus*, 1, 8, 48; *Pseudomonas*, 7, 10, 46; *Salmonella*, 4, 13, 39; *Sarcina*, 5, 11, 46; *Serratia*, 3, 3, 48; *Shigella*, 1, 3, 46; *Staphylococcus*, 3, 9, 46; *Streptococcus*, 4, 4, 57. Of 96 strains in a special study only 16 showed any variation from the original characterization in glucose, lactose, and sucrose fermentation, and in litmus milk reactions. This method of preserving cultures is practical.

A CUP PLATE ASSAY FOR BACITRACIN.

Donald A. Hoff, Alfred R. Stanley, and Ralph E. Bennett, Research Department, Commercial Solvents Corporation, Terre Haute, Indiana.

A rapid, simple method of assay was needed for the investigation of bacitracin production. The dilution type assay given by Meleney *et al.* was seriously affected by the presence of nutrients and solvents and required Seitz filtration of specimens.

The assay devised gave heavy plate growth, satisfactory sensitivity, and constant organism response. The zones of inhibition achieved are much sharper than those in the *Staphylococcus aureus* assay for penicillin. Of four sensitive organisms tested, the best results were obtained with a strain of *Micrococcus flavus*, isolated from the air, which gave zones measuring 11 mm in diameter at a potency of 0.1 u per ml, and 21.0 mm at a potency of 4.0 u per ml. The slope of the response curve was 1.9 mm increase per doubling of dose. When seeded and dosed plates were refrigerated for 16 hours, the response curve became 3.2 mm per doubling of dose, thus comparing favorably to the penicillin cup plate assay.

THE ACTION OF PROFLAVINE ON BACTERIOPHAGE MULTIPLICATION: A METHOD FOR

THE STUDY OF INHIBITORS OF VIRUS GROWTH. *Ruth A. C. Foster*, Bacteriological Laboratories, Indiana University, Bloomington, Indiana.

Metabolic inhibitors of bacteriophage growth, as a general rule, show a strictly parallel action on growth of phage and multiplication of bacteria. It has been reported by Fitzgerald, however, that some acridines are potent phage inhibitors in the absence of inhibition of bacterial growth. This observation seemed to be of sufficient interest to warrant reinvestigation with a technique allowing a single cycle of phage infection and liberation to be analyzed. Using the one-step growth technique (Delbrück and Luria), we studied the action of proflavine and atabrin on the multiplication of phage T_2 on *Escherichia coli*, strain B, in a glucose salt medium. It was found that increasing concentrations of proflavine cause a progressive reduction of phage yield per cell, followed by a complete suppression of liberation. Dilution removes the inhibitory action, provided the exposure of the infected bacteria to the proflavine has not been continued too long. Antagonism of proflavine can be produced by nucleic acids, as previously reported, and by other substances that serve as nutrients. Since it was found that the concentrations of drug which inhibit phage growth and bacterial growth differ merely by a factor of two, it was concluded that this group of substances does not offer much promise as specific virus inhibitors.

RAT BITE FEVER DUE TO STREPTOBACILLUS MONILIFORMIS. *Edith Haynes*, Indiana University Medical Center, Indianapolis, Indiana.

Streptobacillus moniliformis was cultured from the blood of three children with rat bite fever. The blood of each patient was examined by dark-field for *Spirillum minus*, but this organism was not found. Guinea pigs and mice inoculated with blood of the patients were examined at intervals over a period of several weeks for spirilla, but none were found. These patients were treated with penicillin. Two of them made a prompt and complete recovery. The third, after discharge from the hospital, continued to have an elevated temperature and arthralgia for 3 weeks. The following month there was no fever and only occasional episodes of pain in the arms and legs. This patient had not been under treatment with penicillin so long as the two earlier cases.

HISTOPLASMOSIS. *C. G. Culbertson*, Eli Lilly Company, Indianapolis, Indiana.

THE NONFRUITING MYXOBACTERIA. *R. Y. Stanier*, Indiana University, Bloomington, Indiana.

EASTERN PENNSYLVANIA CHAPTER

ONE HUNDRED AND NINETY-SECOND MEETING, PHILADELPHIA
COUNTY MEDICAL SOCIETY BUILDING, FEBRUARY 25, 1947,
PHILADELPHIA, PENNSYLVANIA

RELATION OF DIET TO POLIOMYELITIS IN MICE. *J. H. Jones and C. Foster*, Departments of Physiological Chemistry and Pediatrics and the Children's Hospital, University of Pennsylvania, Philadelphia, Pennsylvania.

STUDIES ON THE SUSCEPTIBILITIES TO TYPHUS OF RATS ON DEFICIENT DIETS. *Florence K. Fitzpatrick*, Virus Department, Medical Research Division, Sharp and Dohme, Glenolden, Pennsylvania.

Studies aimed at correlating diet and susceptibility to typhus are being conducted in an attempt to explain the high mortality which occurs when this disease attacks malnourished populations. Weanling rats are being subjected to various deficient diets for periods of 6 weeks. The diets are planned, not to produce drastic deficiencies, but rather to mimic dietary conditions as they might exist in different parts of the world. It has been found to date that diets low in protein, or low in vitamins of the B complex, or both, render rats much more susceptible to murine typhus than are controls on a complete diet. In paired feeding experiments, curtailment of the B complex vitamins greatly increased susceptibility. Experiments are under way in which one member of the complex at a time is reduced to $\frac{1}{20}$ optimal level. A comparison of natural and synthetic diets will be made, and the effect of low calorie consumption will be studied.

CONSTITUTIONAL PHYSIOLOGY AS SEEN IN INSANITY. *S. de W. Ludlum*, Department of Psychiatry, Graduate School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.

WASHINGTON BRANCH

WASHINGTON, D. C., FEBRUARY 25, 1947

RICKETTSIOSTATIC EFFECT OF STREPTOMYCIN IN EXPERIMENTAL INFECTIONS. *Joseph Smadel, Elizabeth Jackson, and Ross Gauld*, Army Medical School, Washington, D. C.

Streptomycin had rickettsiostatic effect in embryonated eggs with *R. prowazeki*, *R. mooseri*, or *R. rickettsi*, but none with *R. orientalis* in the doses employed Amounts as small as 1.0 mg per egg produced significant effect, although actual prolongation of life was not great. Administration of 10 mg per egg resulted in a distinct lengthening of life of embryos infected with three susceptible organisms.

Experiments using streptomycin of varying purity and other experiments in which bacteriostatic activity of the drug was reduced by semicarbazide indicated antibacterial and antirickettsial effect went hand in hand. Most striking prolongation of life was obtained in eggs treated with 10 mg streptomycin and 0.5 mg *para*-aminobenzoic acid. Ten mg streptomycin and 0.5 mg nitroacridine also displayed a synergistic effect with two of the organisms.

Neither tryptophane nor folic acid and its related compounds had any demonstrable effect on the growth of rickettsiae in embryonated eggs.

SOME POSTWAR PROBLEMS IN TESTING DISINFECTANTS. *L. S. Stuart*, Insecticide Division, Livestock Branch, U. S. Department of Agriculture, Beltsville, Maryland.

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